# THE TRANSCRIPTIONAL REGULATION OF STEM CELL DIFFERENTIATION PROGRAMS BY HEDGEHOG SIGNALLING

By

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## Abstract

The Hedgehog (Hh) signalling pathway is one of the key signalling pathways orchestrating intricate organogenesis, including the development of neural tube, heart and skeletal muscle. Yet, insufficient mechanistic understanding of its diverse roles is available. Here, we show the molecular mechanisms regulating the neurogenic, cardiogenic and myogenic properties of Hh signalling, via effector protein Gli2, in embryonic and adult stem cells.

In Chapter 2, we show that Gli2 induces neurogenesis, whereas a dominant-negative form of Gli2 delays neurogenesis in P19 embryonal carcinoma (EC) cells, a mouse embryonic stem (ES) cell model. Furthermore, we demonstrate that Gli2 associates with *Ascl1/Mash1* gene elements in differentiating P19 cells and activates the *Ascl1/Mash1* promoter *in vitro*. Thus, Gli2 mediates neurogenesis in P19 cells at least in part by directly regulating Ascl1/Mash1 expression.

In Chapter 3, we demonstrate that Gli2 and MEF2C bind each other's regulatory elements and regulate each other's expression while enhancing cardiomyogenesis in P19 cells. Furthermore, dominant-negative Gli2 and MEF2C proteins downregulate each other's expression while imparing cardiomyogenesis. Lastly, we show that Gli2 and MEF2C form a protein complex, which synergistically activates cardiac muscle related promoters.

In Chapter 4, we illustrate that Gli2 associates with *MyoD* gene elements while enhancing skeletal myogenesis in P19 cells and activates the *MyoD* promoter *in vitro*.

Furthermore, inhibition of Hh signalling in muscle satellite cells and in proliferating myoblasts leads to reduction in MyoD and MEF2C expression. Finally, we demonstrate that endogenous Hh signalling is important for MyoD transcriptional activity and that Gli2, MEF2C and MyoD form a protein complex capable of inducing skeletal muscle-specific gene expression. Thus, Gli2, MEF2C and MyoD participate in a regulatory loop and form a protein complex capable of inducing skeletal muscle-specific gene expression.

Our results provide a link between the regulation of tissue-restricted factors like Mash1, MEF2C and MyoD, and a general signal-regulated Gli2 transcription factor. We therefore provide novel mechanistic insights into the neurogenic, cardiogenic and myogenic properties of Gli2 *in vitro*, and offer novel plausible explanations for its *in vivo* functions. These results may also be important for the development of stem cell therapy strategies.

## Acknowledgements

First and foremost I would like to thank my supervisor, Dr. Ilona Skerjanc, for taking the risk of accepting me as an international student to be her trainee. Your guidance was invaluable and the independence was the most cherished experience of all.

I am indebted to many funding organizations, which have looked beyond my citizenship and supported my journey in graduate school: University of Ottawa, Heart and Stroke Foundation of Canada, Ontario Government and Fisher Scientific. I am honoured with the trust and have tried to use this financial support to focus on my projects and advance them as much as I could.

I would like to express my gratitude to numerous collaborators, including Drs. Jean Francois Couture, Nadine Wiper-Bergeron, Alex Blais and Valerie Wallace. I would also like to thank my thesis advisory committee members, Drs. Marjorie Brand and Valerie Wallace. I was privileged to have worked with you and your input was invaluable in my growth as a scientist.

I am wholly grateful to my all of my lab mates. While I started off as a trainee with minimal to no expertise in molecular biology and tissue culture areas; now, with the training by my colleagues, I can set up a 96-well PCR plate in 15 minutes with my eyes closed (or close to it on an early Monday morning) and manually prepare up to 80 plates of mES cell hanging drops.

I would like to take the opportunity and sincerely thank all my mentors, colleagues and students, for their moral and technical support and for countless brainstorming coffee breaks: Drs. Tammy Ryan, Ashraf Al Madhoun, Josee Coutu, Jennifer Dawson, Emeka Enwere, Evelin Loit, and Leslie Mitchell as well as Virja Mehta, Flavia Sendi-Mukasa, Joel Fair, Michael Shelton, Erin Coyne, Jacob Wong, Donna Clary, Jun Liu, Mila Tepliakova, Anna Fischer and Vanja Avdic. Many of you have become dear friends and my new acquired family in Canada. Thank you.

Personally, I would like to thank, from the bottom of my heart, my family: my Mom, Dad and sister. You have been so supportive even thousands of miles away. I am especially grateful for my Mom for her perseverant focus on my education and the sacrifices she made along the way. Mom, while your dreams of me being a musician may have to wait; you made it possible for me to achieve my dreams of becoming a scientist. Thank you.

I would like to end this very and only emotional part of my thesis with words of gratitude for my supervisor, Ilona Skerjanc, and my partner and best friend, Jason A. Tetro. Ilona, thank you for constant support and guidance as a successful woman in science. Jason, thank you for introducing me to Canadian life and family, for your constant support and lastly, for our exquisite scientific discussions at the dinner table, they are truly the highlight of my days. When I came to Canada, I thought I gained a way out. But with you two, I really gained a new way into the world of science and into my new life. I love it. Thank you all so very much.

# Dedication

Dedicated to the first scientist I met; my Mother, Ljubov Smirnova.

# Table of Contents

## List of Abbreviations

ANF - atrial natriuretic factor

ANOVA - analysis of variance

AP – anteroposterior

Ascl1 - achaete-scute complex homolog 1

AV - atrioventricular

bHLH - basic helix-loop-helix

BMP - bone morphogenetic protein

BraT - Brachyury T

CBP - calmodulin binding protein

CER – core enhancer region

ChIP - chromatin immunoprecipitation

DAVID - Database for Annotation, Visualization, and Integrated Discovery

Dbx – developing brain homeobox

Dhh – Desert Hedgehog

DKO – double knockout

DMSO - dimethyl sulfoxide

DRR – distal regulatory region

DV – dorsoventral

E – embryonic day

EB – embryoid body

EC – embryonal carcinoma

ES – embryonic stem

FDA – Food and Drug Administration

FGF - fibroblast growth factor

FHF – first heart field

GATA-4 - GATA-binding protein 4

GFAP - glial fibrillary acidic protein

GFP – green fluorescent protein

Gli - glioma associated

Hand1 - heart and neural crest derivatives expressed transcript 1

HD – homedomain

Hh – Hedgehog

HH - Hamburger–Hamilton stage

HSD - honestly significant difference

IF - immunofluorescence

Ihh – Indian Hedgehog

IP - immunoprecipitation

Irx - iroquois homeobox

Isl1 – Islet-1

kb - kilobase

KO – knockout

MAPK - mitogen-activated protein kinase

MEF2 - myocyte enhancer factor 2

Meox - mesenchyme homeobox

Mesp - mesoderm posterior homolog

MHC – myosin heavy chain

MLCK - myosin light chain kinase

MRF - myogenic regulatory factor

Mulan - Multiple Sequence Local Alignment and Visualization tool

Myf5 – myogenic factor 5

MyoD - myogenic differentiation 1

MyoG – myogenin

NF68 – neurofilament 68

Nkx - NK transcription factor related

OFT – outflow tract

Pax – paired homeobox

PMSF – phenylmethanesulfonylfluoride

PRR – proximal regulatory region

Ptch1 – Patched 1

PVDF - polyvinylidene fluoride

QPCR - Quantitative Polymerase Chain Reaction

RA – retinoic acid

SC – satellite cell

SHF – second heart field

Shh – Sonic Hedgehog

Smo – Smoothened

SynoR - Genome miner for synonymous regulation

TA – tibialis anterior

TAP - tandem affinity purification tag

Tbx - T-box

TF – transcription factor

TSS – transcriptional start site

Tuj1 - β-III tubulin

Wt – wild type

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## Contributions of collaborators

Chapters 2 and 3 are published manuscripts. The author contributions are as follows:

In Chapters 2, 3 and 4 the P19[Gli2] and P19[Gli/EnR] cell lines were generated by Helen Petropoulos, Peter Gianakopoulos, Xionan Wang and Ashraf Al Madhoun.

In Chapter 2, I differentiated parental P19 cells and performed subsequent gene expression analysis, performed and analyzed chromatin immunoprecipitation (ChIP) assay with Gli2 and IgG antibodies from differentiating P19[Gli2] cells, differentiated and analyzed P19[Gli2] cells using QPCR, Western Blot and immunofluorescence (IF) analysis, analyzed QPCR results from P19[Gli/EnR] cells and performed cell counting for all experiments. Anna Fischer participated in the optimization and development of the ChIP assay with Gli2 antibodies. Tammy Ryan performed differentiation of P19[Gli/EnR] cells and subsequent gene expression analysis.

In Chapter 3, I carried out mouse embryonic stem cell, parental and stable P19 EC differentiation, IF, Western Blot, QPCR analysis, ChIP assay with Gli2 antibodies; QPCR analysis of ChIP with Gli2 and MEF2C antibodies, immunoprecipitation (IP), reporter assays, bioinformatics analysis and cell counting for all figures. Ashraf Al Madhoun created and differentiated P19[TAP] and P19[MEF2C-TAP] stable cell lines and performed ChIP assay with MEF2C antibodies. Anna Fischer participated in differentiation of P19[Gli2] cells and ChIP assay with Gli2 antibodies. Michael Shelton participated in QPCR analysis of mouse embryonic stem cells. Christina Karamboulas differentiated P19[Nkx-MEF2C/EnR] cells, performed Northern Blot and IF analysis of this cell line.

In Chapter 4, I differentiated parental P19 and stable P19[Gli2] and P19[Gli/EnR] cell lines, contributed 2 out of 3 replicas for ChIP assay with Gli2 antibodies, performed QPCR and IF analysis for P19 and satellite cells, carried out X-gal staining, treatment and culture of isolated satellite cells, myogenic conversion assays and reporter assays with or without KAAD-cyclopamine or Gli/EnR, reporter assays in P19 cells, IP and bioinformatics analysis. Erin Coyne analyzed ChIP-QPCR assay with Gli2 antibodies, performed reporter assays in C3H10T1/2 cells with MyoD promoter and contributed to muscle cell counting in P19[Gli2] and P19[Control] cell lines. Ashraf Al Madhoun participated in satellite cell *in vitro* expansion. Joel Fair contributed 1 replica of the ChIP assay with Gli2 antibodies. Nadine Wiper-Bergeron, Catherine St-Louis and Grace Li isolated satellite cells from mice. Valerie Wallace and Sherry Thurig supplied Ptch1/LacZ heterozygous and wild type mice.

I hereby confirm that I have contributed more than 50% of experimental work to each chapter, and I have written all chapters with assistance from Ilona Skerjanc.

# CHAPTER 1

# INTRODUCTION

## 1.1 EMBRYONIC STEM AND EMBRYONAL CARCINOMA CELLS

Pluripotent embryonic stem (ES) cells derived from the inner cell mass of the blastocyst hold a tremendous potential to be used in the repair of failing organs through stem cell therapy. ES cells can maintain their pluripotency when cultured for a prolonged period of time *in vitro*, and can differentiate into a multitude of cell types. However, before ES cells can be used in a therapeutic setting, researchers have to tackle several obstacles, such as the enrichment of a desired cell type, elimination of undifferentiated cells and determination of the best cell (progenitor or fully differentiated cell) to repair damaged tissue (1, 2).

The first stem cells of teratocarcinoma origin were isolated over 50 years ago (reviewed in (3)) and were shown to sporadically differentiate into various cell types including muscle, cartilage, bone, skin and neurons *in vivo* (4), and upon formation of embryoid bodies (EBs) *in vitro* (5). Mouse and human ES (mES and hES) cells were first isolated in 1981 (6, 7) and 1998 (8), respectively. Their differentiation into cardiac muscle cells and neurons *in vitro* was achieved relatively early on (9, 10). However, it was not until 1994 when mES cells were shown to differentiate into skeletal muscle cells (11), and until now, there have been only three reports of successful differentiation of hES cells into fully differentiated skeletal muscle *in vitro* (12-14). It is therefore not surprising that researchers took advantage of embryonal carcinoma (EC) cells, which cannot be used in therapeutic applications due to their tumorigenic potential (15), but serve as an excellent stem cell model

system to study a variety of differentiation processes, including cardiac and skeletal myogenesis, as well as neurogenesis *in vitro*.

One of the most studied EC cells is the P19 line. P19 EC cells were originally isolated from induced teratocarcinoma of male C3H/He mouse testes by injection of isolated embryonic day (E) 7.5 mouse embryo cells (16). These cells have stable diploid karyotype (16) and can contribute to tissues in live-born chimeric mice (15). When P19 cells are grown in a monolayer in undifferentiated state, they express stem cell pluripotency markers such as SSEA1, Oct-3/4 and Nanog (17-20). Moreover, undifferentiated P19 cells have a short G1 phase (21) and are characterized by the expression of cyclin D-CDK2/4/6 complexes (22, 23), features known to distinguish the cell cycle of both mES and hES cells (23-25). In tissue culture, aggregation of P19 cells in the absence of chemical stimuli leads to the differentiation of fibloblast-like cells, but myogenesis and neurogenesis do not occur (26, 27). P19 cells aggregated in the presence of dimethyl sulfoxide (DMSO) differentiate into cardiac and skeletal muscle cell types (28). Cardiac muscle appears on day 6 and represents about 15% of the population, whereas skeletal muscle does not appear until day 9 following treatment and represents about 5% of the population (28). P19 aggregates treated with retinoic acid (RA) differentiate into neurons and glial cells (29). Neurons appear on day 6, representing up to 70% of population, whereas astrocytes appear on day 7-10 of differentiation (26). Thus, P19 cell differentiation can be readily controlled and thus provides an easily manipulable system to examine early neurogenesis as well as cardiac and skeletal myogenesis in an embryonic stem cell model system.

The differentiation of mouse and human ES and EC cells follows the hierarchical set of signals and gene expression patterns observed during embryonic development in the

generation of the three germ layers, endoderm, mesoderm and ectoderm (8, 20, 28-31). Therefore, it is important to understand embryonic tissue formation *in vivo*.

## 1.2 NEUROGENESIS

### 1.2.1 Formation of neural tube

The brain and spinal cord are members of the central nervous system (CNS) and develop through a process called neurulation from ectoderm folding to form a neural tube. Neurulation begins in the dorsal part of the embryo by thickening of the dorsal surface of the ectoderm to create a neural plate. This leads to the formation of the neural folds, which will migrate and fuse in the midline to create a hollow neural tube. Murine neural tube closure originates at the 5-somite stage, in the future cervical-hindbrain boundary, continues both rostrally and caudally and finishes with closure in the spinal region at the 30-somite stage. The cells at the most dorsal portion of the neural tube become neural crest cells and contribute to several cell populations in the embryo, including the cells of the peripheral nervous system (32, 33).

### 1.2.2 Differentiation of the neural tube

The constituent neuroepithelial cells forming the neural tube differentiate into neurons and glial cells as the neural tube undergoes gross anatomical changes associated with its compartmentalization. The spatiotemporal position of the differentiated neuronal cells in the neural tube confers their function in the resulting CNS organs, including prosencephalon (forebrain) with optic vesicle, mesencephalon (midbrain), rhombencephalon (hindbrain) and spinal cord (32).

First, multipotent neural progenitor cells acquire unique positions in the neural primordium. Then, these progenitors produce progeny that can differentiate into one neuronal cell type – neuron, oligodendrocyte or astrocyte. These committed neuronal progenitors become specified to produce a particular kind of neuron, e.g. a motoneuron or a specific interneuron. The progenitors divide close to the lumen of the neural tube. Once they stop dividing, they migrate out to form the mantle zone, where cells differentiate into neurons and glia. There are 6 types of differentiated dorsal neurons (dl1–dl6) and 5 types of differentiated ventral neurons (v0–v3 + mn) in the developing neural tube (Fig. 1.1 A). The neurons make connections between each other and extend axons away from the lumen, creating a marginal zone. Glial cells (oligodendrocytes and astrocytes) are produced after neurons and they eventually populate the marginal zone by covering neuronal axons with myelin sheaths (32, 34).

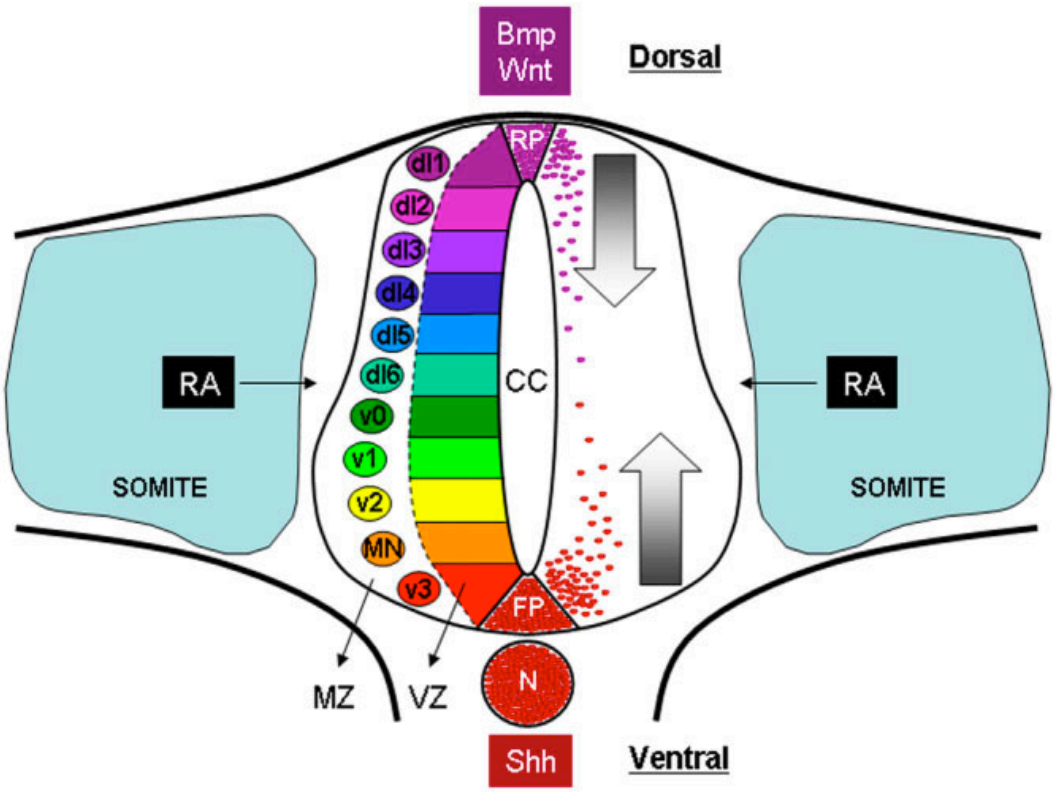
### 1.2.3 Specification of neuronal fates in the neural tube

The spatial specification of neural progenitors along the neural tube lumen and their temporal differentiation into neurons and glia are controlled by the intracellular transcription factors (TFs) and by extrinsic signalling molecules emanating from adjacent embryonic structures.

**Figure 1.1 The role of Shh during neural tube development.**

**(A)** Diagram of a transverse section of the developing spinal cord. Central canal (CC, the lumen of the neural tube) is surrounded by a layer of proliferative progenitor cells in the ventricular zone (VZ). These progenitors differentiate into postmitotic neurons (dI1-6, V0-V2, MN and V3) that are distributed in the mantle zone (MZ). The patterning of progenitors and their derivatives is established along the dorsoventral (DV) axis by the action of gradients like Shh secreted from the notochord (N) and the floor plate (FP), and RA emanating from the adjacent somites. **(B)** Patterning of the ventral spinal cord by the Shh signalling pathway. A Shh gradient is established from the ventral to the dorsal part of the spinal cord, which is translated into an intracellular gradient of Gli activity. Shh graded signalling controls the expression of a set of transcription factors in neural progenitor cells grouped into Class I, repressed by Shh, and Class II whose expression requires Shh. Class I and Class II TFs can repress each other's expression, thereby defining their boundary of expression along the DV axis to establish progenitor domains. Images adapted with permission from (35, 36) from Development and Developmental Dynamics Journals.

**A**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RP
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MN
v3
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CC
RA
RA
SOMITE
SOMITE
MZ
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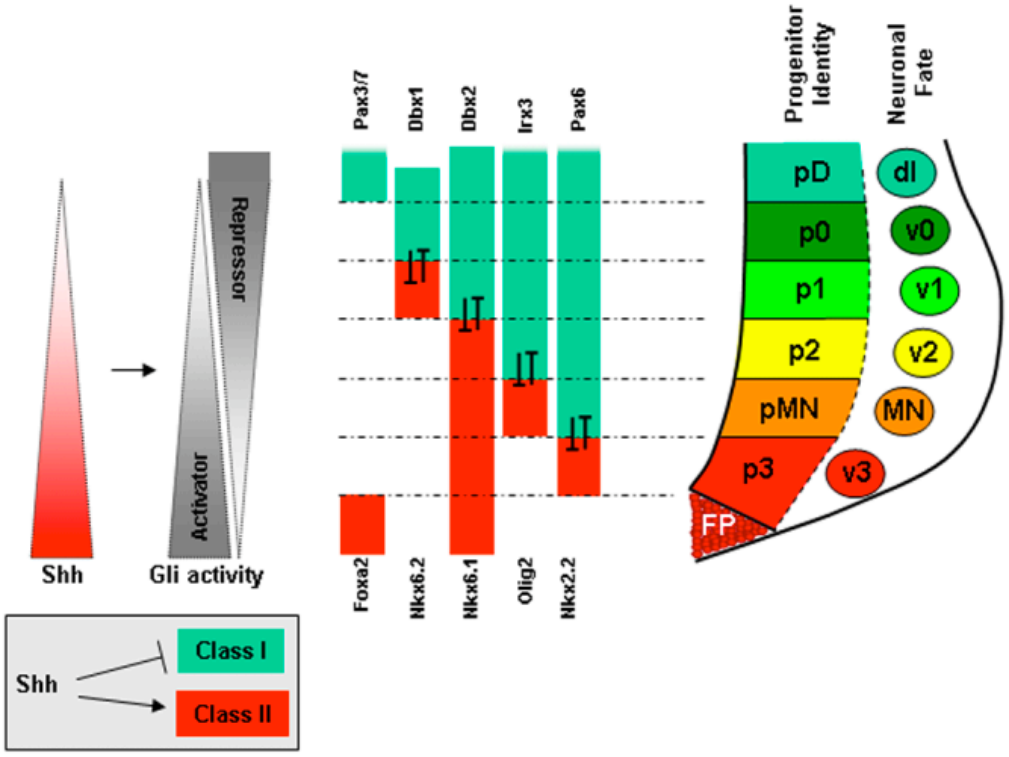
Shh
Ventral

**B**


Pax3/7
Dbx1
Dbx2
Irx3
Pax6
Progenitor Identity
Neuronal Fate
pD
p0
p1
p2
pMN
p3
FP
dI
v0
v1
v2
MN
v3
Foxa2
Nkx6.2
Nkx6.1
Olig2
Nkx2.2
Repressor
Activator
Shh
Gli activity
Shh
Class I
Class II

#### 1.2.3.1 Transcriptional control of neuronal fate specification

Neural progenitor cells in the ventricular zone express a combination of patterning transcription factors that reflect their dorsoventral (DV) positional identity. These factors include homeodomain (HD) factors of the Pax (paired-homeobox), Nkx (NK transcription factor related), Dbx (developing brain homeobox) and Irx (iroquois homeobox) families, as well as basic helix-loop-helix (bHLH) proteins like Olig2 (Fig. 1.1 B). HD proteins of the Otx, Gbx, En and Hox families confer an anteroposterior (AP) positional identity.

Among the best-studied patterning proteins are Olig2, Pax6 and Nkx2-2. Nkx2-2 is important for the formation of the most ventral p3 domain of neural progenitors, which differentiate into V3 interneurons and oligodendrocytes (37, 38). Olig2 is required in the establishment of pMN domain, which differentiates into motoneurons and oligodendrocytes (39, 40). Interestingly, Olig2 and Nkx2-2 physically interact and co-operatively induce oligodendrocyte differentiation (41-43). However, this protein complex can also repress the ability of Nkx2-2 to induce the differentiation of V3 interneurons, suggesting that Olig2 and Nkx2-2 interaction plays a role in the establishment of the boundary between p3 and pMN domains (42). Finally, Pax6 mutant embryos have reduced amount of V2 interneurons and lack V1 neurons entirely (44), whereas the differentiation of V0 interneurons is delayed (45). Thus, Pax6 is involved in the formation of p0-p2 domains that produce V0-V2 interneurons, oligodendrocytes and astrocytes (reviewed in (34)).

When the neural progenitors divide in the ventricular zone, they express progenitor proteins like LIM-HD proteins that determine the fate of the progeny, e.g. the type of neurotransmitter or axonal path in the resulting differentiated neuron. The expression of LIM-HD proteins is known to be regulated by Olig2 (46) and Pax6 (44), therefore suggesting

the diverse roles for HD proteins in establishing 1) the progenitor domains in the ventricular zone; and 2) the identity of the dividing neural progenitors.

Once the progenitor cell has acquired its identity, it starts to express proneural factors (Mash1, Ngn1-3 and Math1). This leads to neuronal commitment, cell cycle exit and differentiation of the neural progenitor cells (reviewed in (34)). Mash1 (Ascl1) null mice have severe neurogenesis defects in the ventral telencephalon (47, 48) and perturbed formation of V2 interneurons (49), dl3 and dl5 neurons (50) in the spinal cord. Ngn1 or Ngn2 single knockout (KO) embryos lack cranial sensory ganglia, whereas Ngn1/Ngn2 double knockout (DKO) animals also lack spinal sensory ganglia and a subset of spinal cord neurons (51-53). Ablation of Math1 (Atoh1) results in loss of D1 interneurons (54). In accordance, ectopic Math1, Mash1 and Ngn1/2 can induce neurogenesis *in vivo* (51, 55).

In postmitotic neurons proneural factors induce the expression of neuronal differentiation bHLH factors like NeuroD (NeuroD1) and NeuroM (NeuroD4/Math3), which contribute to the differentiation program. Although NeuroM KO mice exhibit mild defects in neurogenesis, Mash1/NeuroM DKO mice lack tectal, hindbrain neurons and retinal bipolar cells (56). Moreover, the affected cells adopt glial fate (56). NeuroD is also important in the neuronal-versus-glial cell fate decisions during retina development (57). Therefore, vertebrate proneural and neuronal bHLH proteins play highly heterogeneous roles during CNS development by participating in neurogenesis and gliogenesis (58).

Lastly, the sub-type specificity of the differentiated cells is conferred by the expression of the neuronal HD proteins like Hb9 (Mnx1, motoneurons), Chx10 (Vsx2/Hox10, V2 interneurons), En1 (V1 interneurons) and Evx1/2 (V0 interneurons) (reviewed in (34, 59)). The neurogenic and glial differentiation programs are also negatively

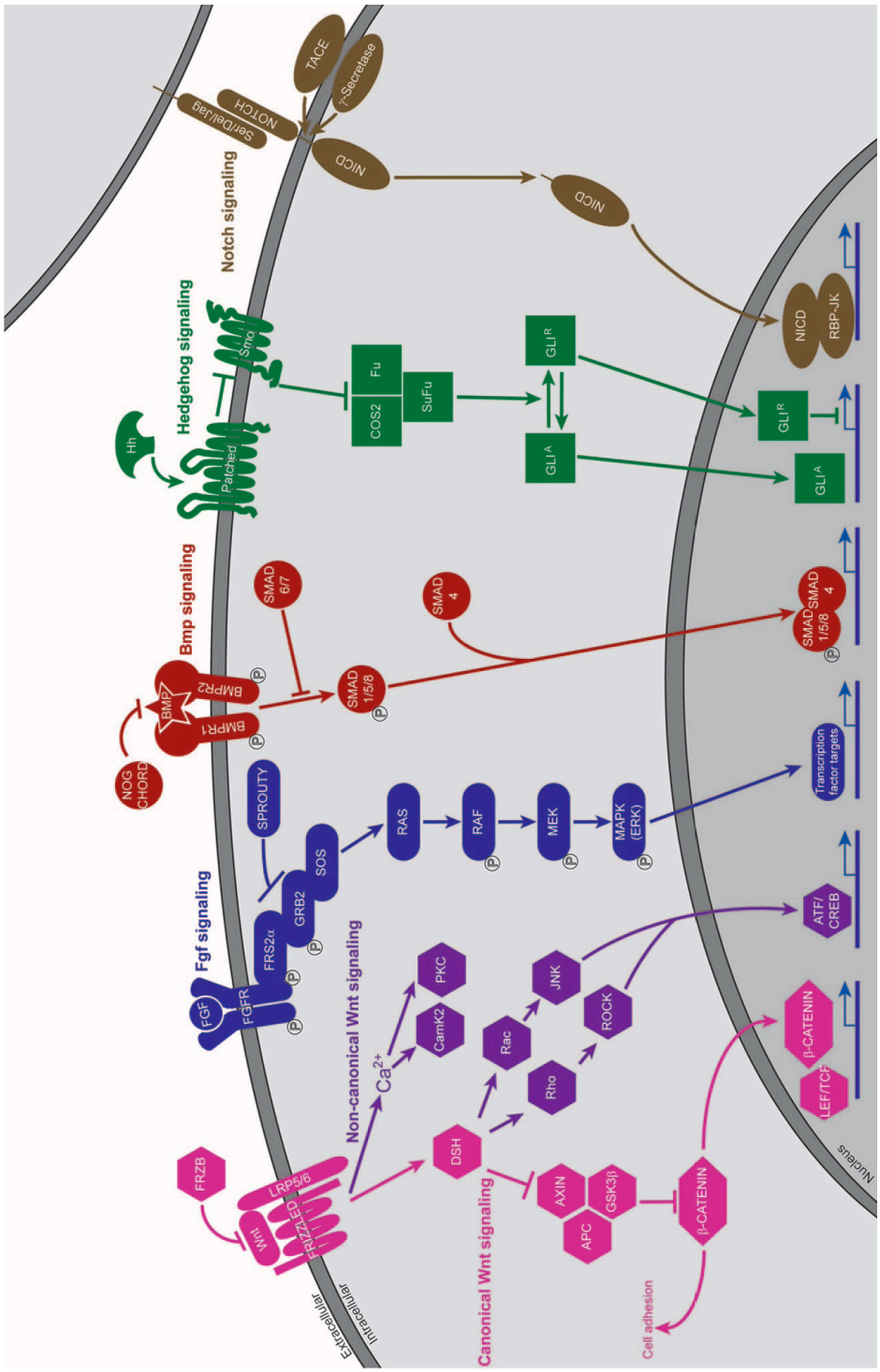
regulated by the inhibitory HLH Id and bHLH Hes proteins, which repress the expression of proneural bHLH proteins and Olig2 (reviewed in (34)).

#### 1.2.3.2 Control of ventral neuronal fate specification by signalling pathways

For the DV patterning to occur, FGF signalling from the presomitic mesoderm (PSM) needs to be switched off. FGF maintains neural progenitors in the caudal zone and regulates the expression of SoxB1 genes (Sox1-3) (reviewed in (60)). Interestingly, SoxB1 genes are inhibitory to neuronal differentiation and must be repressed for neurogenesis to occur (61, 62). However, inhibition of FGF signalling alone does not promote neuronal differentiation in the neural tube and requires somatic retinoic acid (RA) signalling (Fig. 1.1 A) (63, 64). Stimulation of RA signalling in the spinal cord leads to an increased number of NeuroM$^+$ cells (64). In accordance, block of RA synthesis or RA signalling in neural plate explants leads to the reduction of NeuroM$^+$ cells, and vitamin A-deficient quail embryos, which lack all retinoids, demonstrate a reduction of neurons in spinal cord and an absence of Ngn1-2 and NeuroM protein expression (64). Importantly, RA and FGF signalling pathways are antagonistic. It was shown that RA negatively regulates the expression of FGF8 (64, 65), whereas FGF8 represses the expression of Raldh2, RA synthesizing enzyme, both *in vivo* and *in vitro* (64).

**Figure 1.2 Main features of the Wnt, FGF, Bmp and Hh signalling pathways leading to target gene activation.**

Canonical Wnt signalling: Wnt binds to the Frizzled-LRP5/6 receptor complex, which results in the activation of Disheveled (DSH) and uncoupling of β-catenin from a degradation complex composed of APC, axin and GSK3β. β-Catenin translocates to the nucleus and associates with LEF/TCF to activate target gene expression. Noncanonical Wnt signalling: Activated DSH activates Rac and Rho, which stimulate the function of JNK and ROCK. The activated ATF/CREB complex induces target gene expression. Alternately, Wnt signalling results in the activation of the $Ca^{2+}$/calmodulin-dependent kinase 2 (CamK2) and the protein kinase (PK)C pathways. FGF signalling: Binding of FGF leads to autophosphorylation of the FGF receptor tyrosine kinase and activation of FRS2α/GRB2/SOS complex. SOS activates the small G protein RAS, triggering activation of RAF, MEK and ERK kinases. p-ERK phosphorylates target TFs to activate gene expression. Bmp signalling: Binding of Bmp ligands (members of transforming growth factor TGFβ family) activates Smad1/5/8 proteins, which associate with Smad4. The Smad complex enters the nucleus and activates target gene expression. Hh signalling: In the absence of Hh ligand, the Ptch1 receptor inhibits activity of Smo. The Cos2/Fu/SuFu (Costal-2/Fused/Suppressor of fused) complex converts the TF Gli into a repressor form (Gli-R). Binding of Hh ligand to Ptch1 releases the inhibition of Smo. Smo blocks the Cos2/Fu/SuFu complex, leading to formation of Gli active form (Gli-A) that translocates to the nucleus to drive target gene transcription. Notch signalling: Interaction of the Notch receptor with transmembrane ligand proteins Ser/Del/Jag in a neighboring cell triggers the release and translocation of NICD into the nucleus to activate target genes. Adapted with permission from (66).


Notch signaling
TACE
γ-Secretase
NOTCH
Ser/Del/Jag
NICD
NICD
NICD
RBP-JK
Hedgehog signaling
Hh
Patched
Smo
Fu
COS2
SuFu
GLI^R
GLI^A
GLI^R
GLI^A
Bmp signaling
NOG
CHORD
BMP7
BMPR1
BMPR2
SMAD 6/7
SMAD 1/5/8
SMAD 4
SMAD 1/5/8
SMAD 4
Fgf signaling
FGF
FGFR
FRS2α
GRB2
SOS
SPROUTY
RAS
RAF
MEK
MAPK (ERK)
Transcription factor targets
Non-canonical Wnt signaling
Ca2+
PKC
CamK2
Rac
Rho
JNK
ROCK
ATF/ CREB
FRZB
Wnt
LRP5/6
FRIZZLED
DSH
Canonical Wnt signaling
AXIN
APC
GSK3β
β-CATENIN
β-CATENIN
LEF/TCF
Cell adhesion
Extracellular
Intracellular
Nucleus

Sonic Hedgehog (Shh) is produced in the notochord and ventral floor plate (Fig. 1.1 A) and is important for neural tube development. In mammals, Hedgehog (Hh) ligands, including Shh, Indian Hh (Ihh) and Desert Hh (Dhh), bind to the transmembrane receptor Ptch1. This leads to the de-repression of Smoothened (Smo) activity and subsequent activation of Gli (glioma associated) transcription factors. In the absence of Shh, Ptch1 blocks the activity of Smo leading to the formation of Gli repressor forms (Fig. 1.2) (reviewed in (67)). Gli1 appears to be solely an activator and its expression is dependent on Gli2 and/or Gli3 (68, 69), whereas Gli2 is mainly an activator, and Gli3 is mainly a repressor (67).

Shh is produced in the notochord and acts in a concentration-dependent manner to induce ventral cell types in the developing neural tube (Fig. 1.1 A). The quantification of Shh-GFP protein, produced by the insertion of GFP coding sequence into the Shh endogenous locus, revealed that the gradient of Shh-GFP protein decays exponentially along the DV axis with the highest expression detected in the most ventral floor plate and p3 domain (70). The neural progenitor cells interpret the graded Shh signalling by regulating the activity of Gli TFs, which in turn regulate the expression of the patterning HD and bHLH proteins, which are subdivided into two classes depending on their mode of regulation. Class I proteins like Pax3/7, Pax6, Irx3 and Dbx1/2 are repressed by Shh, and thus the ventral limit of their expression is determined by the Shh signalling pathway. Class II proteins like Olig2, Nkx6-1 and Nkx2-2 are induced by Shh and their dorsal limit of expression is defined by graded Shh signalling. Furthermore, class I and II genes are mutually repressive, thus contributing to the establishment of boundaries between different progenitor domains (Fig. 1.1 B) (60).

Gli1, Gli2 and Gli3 are expressed in the developing neural tube (71) and can all induce neurogenesis when expressed ectopically (71-73). Gli1 seems to be solely an activator and is dispensable for normal mouse development (69, 74). In contrast, Gli2 mutant mice lack floor plate and exhibit reduced formation of V3 interneurons, while motoneurons are expressed more ventrally (75). This phenotype can be rescued with the expression of Gli1 in place of Gli2, suggesting that Gli2 acts as an activator during neural tube patterning (76). In Gli3 mutant mice, the patterning of the intermediate region of the neural tube is disrupted, but can be rescued by the expression of a truncated allele that encodes the Gli3 transcriptional repressor (77). The role of Gli3 as a repressor is further evidenced by the analysis of embryos that lack both Shh and Gli3. In $Shh^{-/-}$ mice, there is a complete absence of floorplate, p3 and pMN domains and a severe reduction and ventral displacement of p0, p1 and p2 domains (78). However, Shh/Gli3 DKO mice have recovered generation of motoneurons and V0, V1 and V2 interneurons, indicating that Gli3 represses ventral neuronal fates in the absence of Shh signalling (79). The failure to recover the formation of floor plate and V3 interneurons in Shh/Gli3 DKO mice (79) suggests that active Hh signalling is required to induce these cell types. In accordance, in $Ptch1^{-/-}$ mice, where negative regulation of the Shh signalling is removed, dorsal progenitor identities are absent except for the roofplate, and most of the cells adopt floorplate identity along the DV axis of the neural tube (80). Thus, Shh signalling regulates the formation of floorplate, V0-V3 interneurons as well as the specification of the motor neuron progenitor identity (Fig. 1.1 B).

The development of ventral neural progenitors does not solely depend on Shh. For example, V0 and V1 interneurons, which are not absent from the Shh KO mice (78), depend on RA signalling (81). Moreover, class I genes are positively regulated by RA and negatively regulated by FGF signalling (64). Class II Shh-induced genes are also negatively regulated

by FGF signalling (82). Furthermore, Wnt signalling molecules, produced by the roofplate (Fig. 1.1 A), have been implicated in the direct regulation of class II proteins, as well as in the induction of Gli3 expression (reviewed in (36)). Wnt molecules bind to Frizzled-LRP5/6 receptors eventually leading to the stabilization and nuclear translocation of β-catenin, where it interacts with Tcf/Lef TFs to induce gene expression (Fig. 1.2). The deletion of Tcf binding sites in the Nkx2-2 enhancer leads to the more dorsal expression of the reporter as compared to the control, whereas the deletion of the Gli binding site abolishes its expression entirely (83). When stabilized β-catenin is expressed in the Olig1 ventral spinal cord domain, it induces V2 interneuron identity at the expense of motoneurons. This phenotype can be partially rescued with the removal of Gli3, but not Gli2 (84). Interestingly, the repressor form of Gli3 binds and directly inhibits active β-catenin (85), and can also interact with Smad proteins (86), which are effector TFs of BMP signalling molecules (Fig. 1.2) expressed in the roofplate (Fig. 1.1 A). Therefore, the DV boundaries of the neural progenitor domains are established by an intricate and still poorly understood crosstalk between Shh, Wnt, BMP, FGF and RA signalling pathways.

### 1.2.4 Regulation of neurogenesis in stem cells

The differentiation of both ES and EC cells with RA follows the differentiation timeline *in vivo*, where the formation of neurons precedes that of the glial cells (26, 87). Application of RA to forming EBs in suspension or to cells grown in monolayer causes the cells to express markers like Sox1, Mash1, Math1 and NeuroD (88-95), eventually leading to the formation of neurons expressing various neurotransmitters and ion channels (18, 29, 94). When RA treatment is combined with exogenous Shh, mES cells differentiate into motoneurons, which are capable of forming functional connections with muscle fibers *in*

*vitro*, and when transplanted into chick neural tube, are also able to connect with appropriate muscle groups (93, 96). The overexpression of Shh in hES cells enhances neurogenesis and drives the differentiation of neural progenitors into motoneurons (97). Microarray analysis of differentiating hES cells overexpressing Shh reveals an upregulation of genes involved in neural development and specification, such as NeuroD, and class II genes like FoxA2, Nkx2-2 and Nkx6-1. A class I gene Pax3 and an inhibitory gene Id1 are downregulated by Shh (97). Gli1 directly binds gene regulatory elements of several genes including Nkx2-2, Nkx2-9 and FoxA2 and enhances levels of ventralized neurons in mES cells (98). Moreover, Gli1 enhances the expression of proneural and neuronal bHLH factors like Ngn2, NeuroM and Mash1 in differentiating mES cells (98). Gli2 is important for neural progenitor proliferation through direct regulation of pluripotency genes Sox2 and Nanog (99, 100). Finally, Gli1-3 induce, whereas Gli1-3 morpholinos repress, the expression of NeuroD and Ngn1 in *Xenopus* animal caps (71-73).

P19 cells overexpressing Mash1, NeuroD or Ngn1 undergo efficient differentiation into functional neurons, but not glial cells, in the absence of RA or cellular aggregation (101). Moreover, Mash1 alone or in combination with Brn2 (Pou3f2) and Myt1l converts fibroblasts into neurons (102). Interestingly, Ngn1 promotes neural differentiation, but inhibits gliogenesis in embryonic cortical stem cells (103). In accordance, Mash1 and Ngn1-3 promote neurogenesis in neurosphere cultures established from E13.5 rat ventral spinal cords (58). Furthermore, only Mash1 can drive oligodendrogenesis in these neurospheres (58). However, when proneural bHLH factors are co-expressed with patterning genes Olig2 and Nkx2-2, they exhibit elevated formation of oligodendrocytes (58).

Thus, the Shh signalling pathway participates in the direct regulation of class II genes and pluripotency transcription factors in neural progenitor cells. The expression of proneural

bHLH factors, which play an important role during neurogenesis and gliogenesis in stem cells, can also be modulated by Shh signalling. However, the mechanism of this regulation, particularly by Gli factors, remains unknown.

## 1.3 CARDIOMYOGENESIS

### 1.3.1 Formation of the heart

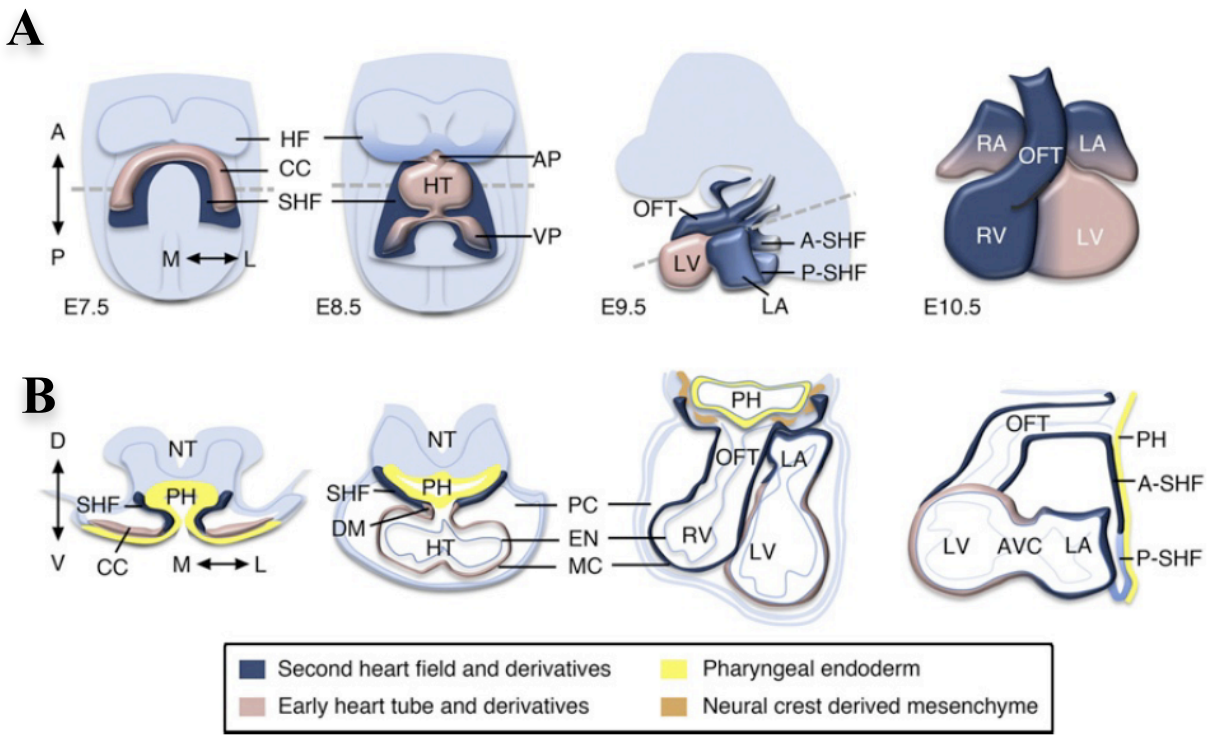
The heart is the first organ to develop and it emerges from mesodermal progenitors, which leave the anterior primitive streak and move laterally to form separate right and left heart forming regions. These progenitor cells then move cranially and form a crescent-like structure under the head folds, which eventually fuses at the embryonic midline to form a beating linear heart tube. The heart tube undergoes gross morphological changes including heart looping, septation and elongation to yield a mature four-chambered heart (104).

### 1.3.2 Differentiation of myocardium *in vivo*

The heart forming regions initiate their differentiation into the endocardial and myocardial lineages when migrating to the midline, which occurs due to the forces generated by the formation of pharynx and the general folding of the embryo. The resulting pre-cardiac mesoderm contains progenitors that contribute to different regions of the heart. The first heart field (FHF) resides in the cardiac crescent and gives rise to the linear myocardial tube, which is destined to become left ventricle, part of the right ventricle and atrioventricular canal. As the heart tube grows, myocardial progenitors that are destined to become atria are added later to the anterior pole of the heart. The second heart field (SHF) is located medially to the FHF and contributes myocardial progenitors to both the anterior and posterior heart poles giving rise to the right ventricle, outflow tract and inflow region (105) (Fig. 1.3).

**Figure 1.3 The formation of the mammalian heart.**

**(A)** Diagrams showing the stages of mouse heart development. From left to right: embryonic day (E) 7.5 (cardiac crescent, ventral view), E8.5 (early heart tube, ventral view), E9.5 (looped heart, left lateral view), and E10.5 (ventral view). SHF progenitor cells and their contribution to the heart are shown in dark blue and the early heart tube (FHF derivative) giving rise to the left ventricle in pink. **(B)** Schematic histological sections showing the location of SHF cells and their myocardial derivatives (dark blue) at the stages shown in (A) relative to pharyngeal endoderm (yellow), early heart tube (pink), and neural crest-derived mesenchyme (orange). The first three panels show transverse sections at the levels indicated by the dotted lines in (A). The right hand panel shows a mid-saggital E9.5 section. Anterior (A), posterior (P), dorsal (D), ventral (V), medial (M) and lateral (L) embryonic axes are indicated. CC, cardiac crescent; SHF, second heart field; HF, head fold; HT, heart tube, AP, arterial pole; VP, venous pole; OFT, outflow tract; LV, left ventricle; LA, left atrium; RV, right ventricle; RA, right atrium; A-SHF, anterior component of the second heart field; P-SHF, posterior component of the second heart field; NT, neural tube; PH, pharynx; PC, pericardial cavity; DM, dorsal mesocardium; EN, endocardium; MC, myocardium. Image adapted with permission from (106).


A
A
P
M
L
HF
CC
SHF
E7.5
HT
AP
VP
E8.5
OFT
LV
A-SHF
P-SHF
LA
E9.5
RA
OFT
LA
RV
LV
E10.5
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NT
SHF
PH
CC
M
L
NT
SHF
PH
DM
HT
PC
EN
MC
PH
OFT
LA
RV
LV
OFT
PH
A-SHF
LV
AVC
LA
P-SHF
Second heart field and derivatives
Early heart tube and derivatives
Pharyngeal endoderm
Neural crest derived mesenchyme

The addition of myocardial progenitors and their differentiation is a continuous process and it eventually leads to the formation of a septated four chambered heart by E14.5 (105).

The differentiation of cardiomyocytes from mesodermal progenitors is regulated by various signalling pathways and transcription factor codes.

#### 1.3.2.1 Transcriptional regulation of cardiomyogenesis *in vivo*

One of the earliest markers of cardiac progenitors is Mesp1/2 (mesoderm posterior 1/2 homolog), which is expressed in the mesoderm adjacent to the primitive streak (107). Ablation of Mesp1 and Mesp2 results in the inability of mesodermal cells to move cranially and create a pre-cardiac mesoderm (108).

The key myocardial progenitor transcripts like Nkx2-5 (NK2 transcription factor related, locus 5), GATA-4 (GATA-binding protein 4), Hand1 (heart and neural crest derivatives expressed transcript 1) and Tbx5 (T-box 5) are first detected in the cardiac crescent. Nkx2-5 knockout mice demonstrate differentiation of cardiomyocytes and formation of a linear heart tube (109). However, looping morphogenesis is not initiated and in addition, Nxk2-5 knockout mice lack ventricular myocardial tissue (109). Hand1 mutants die from extraembryonic defects before the formation of the heart, but the tetraploid chimeric mice show abnormal heart looping and a failure of Hand1 mutant cells to contribute to the left ventricle (110). Notably, the expression of Hand1 is inhibited in Nkx2-5$^{-/-}$ mice both at the cardiac crescent and at the linear heart tube stage, where left ventricle would normally develop (109). GATA-4$^{-/-}$ mice exhibit cardiac bifida, where left and right heart forming regions fail to fuse into a linear heart tube, and generate two independent heart tubes (111). Tbx5$^{-/-}$ mice show deformed linear heart tube with a hypoplastic primitive left ventricle, atria

and inflow tract structures (112). Notably, the expression of GATA-4, Nkx2-5 and its target ANF (atrial natriuretic factor), are severely reduced in the myocardium of the linear tube of $Tbx5^{-/-}$ mice (112). Thus, since the above-described mutant mice show defects in the formation of left ventricle or linear heart tube, which are derivatives of the FHF, it is believed that Nkx2-5, Hand1, GATA-4 and Tbx5 are important during FHF development (104).

LIM-homeodomain protein Isl1 (Islet-1, insulin gene enhancer protein) is one of the earliest SHF markers, as its expression is detected in proliferating SHF progenitors, but not in the heart or differentiated cardiomyocytes (113). Isl1 is a critical early regulator of the SHF development as $Isl1^{-/-}$ mice fail to undergo cardiac looping morphogenesis and lack the OFT (outflow tract) and the right ventricle, derivatives of the SHF (113). Another transcription factor that is expressed in proliferating SHF progenitors is Tbx1, which is both important and sufficient for the OFT myocardium development (114, 115). As SHF progenitors approach the heart tube, the expression of Nkx2-5, GATA-4 and MEF2C (myocyte enhancer factor 2C) is activated (116, 117). MEF2C null mice exhibit defective cardiac looping, a single ventricular chamber and abnormal inflow and outflow tracts (118-120), which is suggestive of the SHF phenotype. The analysis of the expression of LacZ under the regulation of *Mef2c* SHF-specific cardiac enhancer, which requires Isl1 and GATA-4 (119), shows that the distribution of SHF-derived cells in the single ventricle of MEF2C null mice is perturbed (117). Thus, MEF2C appears to be important for the normal addition and distribution of SHF progenitors in the developing heart. MEF2C is also weakly expressed in FHF and recent studies demonstrate the requirement of MEF2C for the proper addition of FHF progenitors to the left ventricle (121). Although the knockout studies

demonstrate the importance of Nkx2-5 in FHF development as discussed above, the *Nkx2-5* gene contains a conserved enhancer that directs the expression of Nkx2-5 in SHF and its derivatives and requires Isl1 and GATA binding elements (122), similarly to the *Mef2c* cardiac enhancer (121). Furthermore, Nkx2-5 is also implicated in the regulation of MEF2C expression in SHF (123). Tbx20, which is important for the development of SHF derivatives (122), synergizes with Isl1 and GATA-4 to drive the expression of Nkx2-5 and MEF2C in the SHF (122). Thus, multiple transcription factors are expressed in both heart fields as demonstrated by the microarray analysis of FHF and SHF murine progenitors from E9.5 (124). The regulation of the cardiomyogenic TFs in different heart fields, as well the regulation of cardiomyogenesis by these TFs, is still under investigation and requires further study.

Surprisingly, no single transcription factor is essential for the differentiation of cardiomyocytes, as documented by numerous knockout phenotypes (reviewed in (104)). It is likely that the compensation arises from different TF groups or from the TFs within the same protein family. Thus, although MEF2C mice still demonstrate the formation of myocardium in a single ventricular heart (118), the expression of dominant-negative MEF2C protein with an engrailed repressor domain under the regulation of *Nkx2-5* enhancer, which results in the repression of all MEF2-dependent genes in cardiomyoblasts, leads to a severely diminished cardiomyogenesis and subsequently, lack of heart development in transgenic mice (125). In accordance, no single transcription factor is responsible for converting non-cardiac muscle cell types into myocardium. This is in contrast with MyoD, a master regulator of skeletal myogenesis (126), and Mash1, a master regulator of neurogenesis (102). Instead, a group of transcription factors, including Tbx5, GATA-4 and MEF2C (127, 128) or Baf60c (129),

were shown to convert cardiac fibroblasts into functional cardiac muscle *in vivo* and/or *in vitro*.

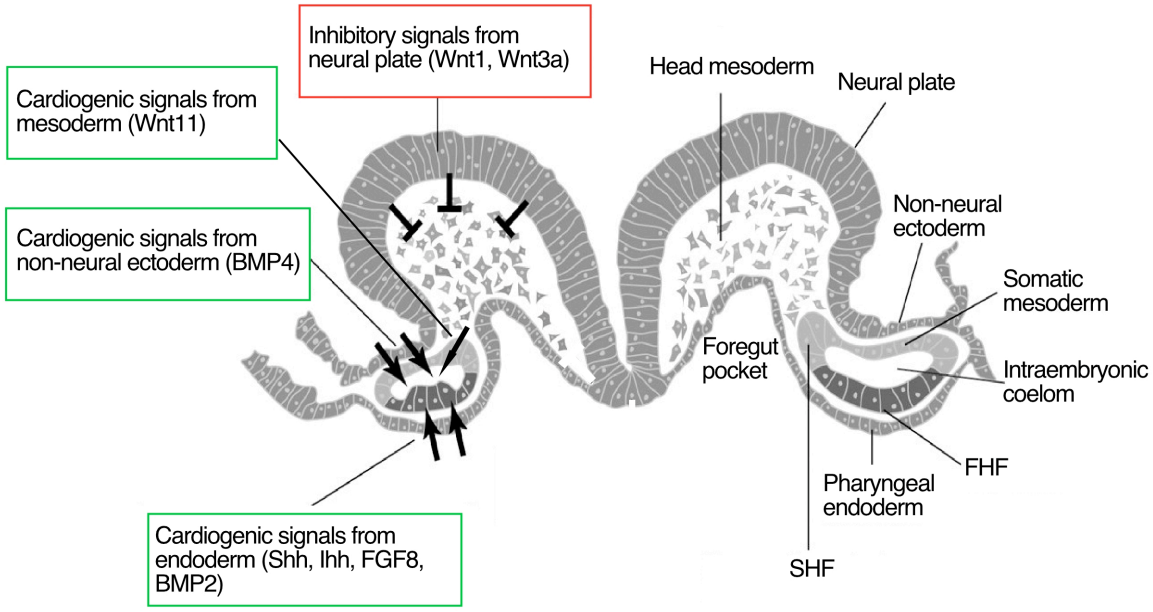
#### 1.3.2.2 The role of signalling pathways during cardiomyogenesis *in vivo*

Cardiomyogenesis *in vivo* is orchestrated by the network of positive- and negative-acting signalling pathways (Fig. 1.4). Positive-acting signals emanating from adjacent tissues include Shh/Ihh, BMP2 and BMP4 (bone morphogenetic protein), FGF-8 (fibroblast growth factor 8), and Wnt11. Negative-acting signals include Wnts 1, 3a and 8 and noggin (Fig. 1.4) (reviewed in (66, 130, 131)).

Removal of the endoderm, a source of FGF and BMP ligands, leads to rapid downregulation of Nkx2-5 and MEF2C expression in chick cardiac mesoderm (132). The ectopic FGF-8 can induce the expression of Nkx2-5 and GATA-4 factors in the regions where BMP proteins are also present (132). Notably, limited exposure of BMP alone fails to upregulate these cardiomyogenic factors and requires FGF signalling (133). During early cardiomyogenesis, BMP signalling is essential for FHF progenitor specification, as ablation of Bmpr1a, a BMP ligand receptor (Fig. 1.2), in Mesp1-expressing cardiac mesoderm, results in the absence of cardiac crescent and primitive ventricle, and blocked expression of Hand1 and Tbx-5 (134). Furthermore, inhibition of BMP signalling by Noggin abolishes precardiac mesoderm differentiation in chick (135). In SHF, BMP2 is expressed in splanchnic mesoderm, caudal to the OFT myocardium, and is important for SHF progenitor differentiation (116). The autocrine FGF signalling, originating from FGF-8 expressed in $Mesp1^{+}$ cardiac mesoderm, is important for the formation of heart tube, looping and accretion of right ventricle and OFT (136). Endodermally-derived FGF-8, however, is important for the OFT septation (136).

**Figure 1.4. Signalling pathways regulate differentiation of pre-cardiac mesoderm.**

Stylized transverse section through the posterior region of the cardiac crescent at E7.5-8.0 of a mouse embryo. Signals, which induce cardiomyogenesis, include BMP2, Shh, Ihh and FGF8 secreted by pharyngeal endoderm, BMP4 secreted by ectoderm and Wnt11 secreted by mesoderm (green boxes, black arrows). Signals that inhibit cardiomyogenesis, include Wnts 1 and 3a, which are secreted by neural plate (red box, black bars). FHF (cardiac crescent, dark grey area) and SHF constitute pre-cardiac mesoderm, which remains in close contact to pharyngeal endoderm. Shh is also expressed in the notochord and Hensen's node, and Wnts 3a and 8 are also expressed in the primitive streak (not shown). Figure is adapted with permission from (130, 137).


Inhibitory signals from neural plate (Wnt1, Wnt3a)
Cardiogenic signals from mesoderm (Wnt11)
Cardiogenic signals from non-neural ectoderm (BMP4)
Cardiogenic signals from endoderm (Shh, Ihh, FGF8, BMP2)
Head mesoderm
Neural plate
Non-neural ectoderm
Somatic mesoderm
Foregut pocket
Intraembryonic coelom
FHF
Pharyngeal endoderm
SHF

Wnt signalling functions via β-catenin (canonical Wnt signalling) or JNK/ROCK kinases and $Ca^{2+}$ signalling pathway (non-canonical Wnt signalling) (Fig. 1.2). Wnt11 is expressed in cardiogenic mesoderm (131) and activates the non-canonical Wnt signalling pathway, whereas Wnts 3a and 8, which are expressed in primitive streak (131) and Wnts 3a and 1, which are expressed in dorsal neuroectoderm (138), activate canonical Wnt/β-catenin pathway (139). Wnt3a is essential for the formation of $Mesp1^{+}$ pre-cardiac mesoderm (140). However, for the specification of pre-cardiac mesoderm to occur, canonical Wnt/β-catenin pathway has to be inhibited, whereas non-canonical Wnt signalling has to be activated, as indicated by the early studies of *Xenopus* heart formation (139). This is confirmed by the studies in higher vertebrates, where mice lacking β-catenin in $Mesp1^{+}$ pre-cardiac mesoderm exhibit the formation of cardiac crescent and FHF progenitors (134). However, these mice have reduced expression of Isl1 and BMP4 in SHF progenitors and are deficient in the formation of the right ventricle and heart looping (134). In contrast, mice with constitutively active β-catenin in $Mesp1^{+}$ pre-cardiac mesoderm lack heart tube, but show expansion of $Isl1^{+}$ and $BMP4^{+}$ SHF progenitors (134). Thus, Wnt/β-catenin signalling regulates SHF development, and the level and/or timing of Wnt/β-catenin signalling is critical for proper heart tube development and looping.

Shh is broadly expressed in the embryo, including midline structures like Hensen's node, notochord and floorplate, as well as gut endoderm (141, 142). The myocardial progenitors of all four heart chambers directly respond to Shh signalling during early heart formation as evidenced by fate-mapping analysis (143). Studies in zebrafish demonstrate the role of Shh signalling for the formation of a proper amount of cardiomyocytes in the developing heart (143). In higher vertebrates, Shh is important for the proliferation of SHF

progenitors (142), and total or endoderm-specific knockout of Shh results in deficient OFT development (144, 145). Total Shh/Ihh DKO results in delayed heart formation and delayed Nkx2-5 expression, similarly to Smo KO phenotype (146). These mice are also deficient in embryo turning, most probably due to the regulation of left/right asymmetry genes by the Hh ligands in the Hensen's node (146). Total knockout of Ptch1, where the negative regulation of Hh signalling is removed, demonstrate elevated Nkx2-5 expression at the cardiac crescent stage (146). These results demonstrate the importance of Hh signalling during FHF development.

Therefore, BMP, FGF, Shh and non-canonical Wnt signalling function to increase the myogenic potential of myocardial progenitor cells, whereas canonical Wnt/β-catenin signalling can exert dual roles during myocardial progenitor formation and differentiation. Although there is some cross talk between BMP and Shh (147) or FGF (133), the nature and extent of the cross-talk between signalling pathways during heart development is still poorly understood.

### 1.3.3 Regulation of cardiomyogenesis in stem cells

ES cell differentiation into cardiac muscle closely resembles early cardiomyogenesis *in vivo*. As ES cells differentiate, the formation of Brachyury T (BraT) positive mesoderm is induced leading to the induction of $Mesp1^{+}$ progenitor cells, which, when isolated, give rise to the FHF- and SHF-specific cardiac progenitors (148). As the FHF and SHF cardiac muscle progenitors differentiate, the expression of Nkx2-5, Tbx5, GATA-4 and MEF2C is induced leading to the formation of beating cardiomyocytes expressing contractile proteins like myosin heavy chain (MHC) and cardiac troponin T (149, 150). Although the molecular signature of FHF- and SHF-derived progenitors derived from ES cells is distinct, these

cardiac muscle progenitor cells share a large set of cardiac-specific transcription factors, similar to the cardiac muscle progenitors in an embryo (124). This suggests that the expression of key cardiac muscle transcription factors in different progenitors may be regulated by a similar subset of signalling molecules.

Many key cardiac transcription factors can induce cardiomyogenesis in pluripotent P19 cells, including MEF2C, Nkx2-5 and GATA-4 (151, 152), whereas their dominant-negative fusion proteins result in ablation of cardiomyogenesis (125, 153). The activity of Nkx2-5 is positively regulated by BMP-4 and negatively regulated by noggin, a BMP signalling inhibitor, in P19 cells (154). The ablation of cardiomyogenesis by noggin in P19CL6 cells, a subclone of P19 cells that stably expresses cardiac α-actin, can be bypassed by forced expression of Nkx2-5 together with GATA-4, but not by Nkx2-5 or GATA-4 alone (155). Thus, the activity of Nkx2-5 is also regulated by GATA-4. Notably, the inhibition of BMP signalling by noggin also results in blocked Shh signalling as assessed by reduced expression of Gli1, Gli2 and Ptch1 in differentiating P19 cells (156). Shh signalling is not essential for cardiomyogenesis in P19 cells, as evidenced by delayed expression of BMP-4 and GATA-4 and subsequently, delayed cardiomyogenesis in P19 cells treated with cyclopamine, a Hh signalling inhibitor (156). The ectopic activation of Shh signalling by overexpression of Gli1, Gli2 or Shh, on the other hand, induces cardiomyogenesis in pluripotent P19 cells (157). Thus, Shh and BMP signalling are positive regulators of cardiomyogenesis in pluripotent cells. In contrast, Wnt signalling during mES cellular cardiomyogenesis exhibits a bi-phasic role, in which activation of Wnt/β-catenin signalling promotes early cardiac differentiation by enhancing Mesp1 expression, but inhibits late stages of cardiomyogenesis, similarly to the embryo (158). Interestingly, Wnt/β-catenin

signalling is important for BMP-4 mediated activation of Smad1 in hES cells (159), thus presenting a possible novel mechanism of positive regulation of cardiac muscle differentiation by the Wnt signalling.

Therefore, key cardiac transcription factors like Nkx2-5, MEF2C and GATA-4 play an important role during cardiomyogenesis in stem cells, similar to the embryo. Signalling pathways exhibit some cross-talk while affecting cardiomyogenesis in stem cells. However, the molecular mechanisms underlying the transcriptional regulation of cardiac TF expression and activity by the signalling pathways both *in vivo* and *in vitro* remain to be fully elucidated.

## 1.4 SKELETAL MYOGENESIS

### 1.4.1 Formation of skeletal muscle *in vivo*

#### 1.4.1.1 Somite formation

Skeletal muscle in vertebrates arises from paraxial mesoderm, which is eventually segmented into somites located on either side of the notochord and neural plate (Fig. 1.5). Rhythmic production of somites from the anterior tip of paraxial mesoderm is controlled by periodic waves of gene expression, which is in turn controlled by gradients of signalling molecules like RA, FGF, Wnt/β-catenin, Notch and Shh (160, 161). The segmentation of the somite is dependent on a molecular clock triggered by Notch and Wnt signalling. The targets of the Notch pathway, Hes1 (hairy and enhancer of split 1) and Lnfg (lunatic fringe), and the Wnt signalling target Axin2, show temporal oscillating expression patterns in the paraxial mesoderm. The periodic segment formation is triggered during a defined, or permissive, phase of the molecular clock, and the clock itself is constantly displaced posteriorly by a

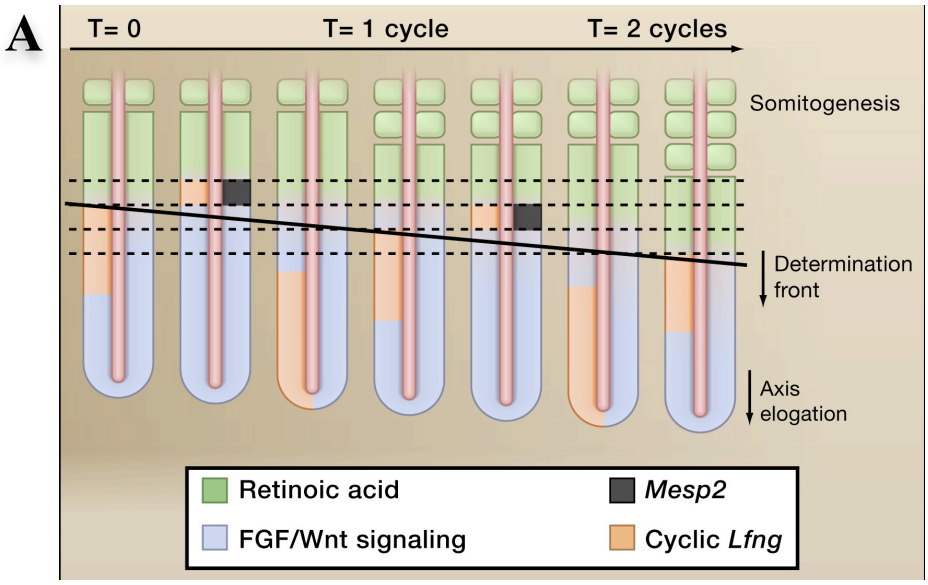
determination front. The determination front is where the opposing signals, FGF and Wnt from tailbud, and RA from the anterior paraxial mesoderm, meet. FGF and Wnt signalling antagonize somitogenesis, whereas RA promotes somite segmentation. The determination front moves posteriorly as the embryo axis elongates and FGF/Wnt mRNA decays. Thus, as the undetermined cells from the posterior end of paraxial mesoderm reach the determination front, they are exposed to waves of oscillating gene expression, creating the periodic clock. This initiates the segmentation program and activates the expression of genes such as Mesp2, which marks the future boundaries of the individual somites (Fig. 1.5 A) (reviewed in (160)). Somite formation is believed to be independent of neural tube and notochord (162), a major source of Shh. However, this model is currently challenged by the work of Resende et al., where authors demonstrate that notochord ablation or inhibition of Shh signalling results in delayed somitogenesis, accompanied by an increase in the molecular clock oscillations, which can be rescued by the exogenous Shh (161).

#### 1.4.1.2 Somite patterning

Once the somite buds off the paraxial mesoderm, it begins the process of patterning (Fig 1.5 B). The dorsal part of the somite becomes dermomyotome, whereas the ventral part of the somite becomes sclerotome. Dermomyotome is further classified into epaxial and hypaxial dermomyotome. As dermomyotome disintegrates, muscle progenitors migrate into the myotome. Cells of the epaxial dermomyotome migrate from the dorso-medial lip adjacent to the neural tube and give rise to deep back muscles. Cells of the hypaxial dermomyotome migrate through the ventral-lateral lip, which lies away from the neural tube and the notochord, and give rise to the rest of the body muscles, including limbs (163).

**Figure 1.5. Somite formation and patterning.**

**(A)** A diagram of somite segmentation. Antagonistic gradients of FGF/Wnt signalling (blue) and RA signalling (green) establish the determination front (solid black line). The periodic signal of the segmentation clock is shown in orange (cyclic Lnfg, represented on the left side only). As the embryo extends, the determination front moves posteriorly. As cells reach the determination front, they are exposed to the periodic clock signal, initiating the segmentation program and activating expression of Mesp2 (black stripes, represented on the right side only) in a stripe domain that prefigures the future segment. This establishes the segmental pattern of the presumptive somites. Adapted with permission from (160). **(B)** Somites are formed from presomitic paraxial mesoderm, which is located on either side of the notochord. During somite patterning, the ventral part of the somite becomes sclerotome and the dorsal part of the somite becomes dermomyotome. Dermomyotome is further subdivided into the hypaxial and the epaxial dermomyotome. Epaxial dermomyotomal cells in the dorsal medial lip (DML) migrate to form the epaxial myotome. Hypaxial dermomyotomal cells in the ventral lateral lip (VLL) migrate to form hypaxial myotome. Cells of the VLL delaminate and migrate to regions of muscle development in the limbs. Adapted with permission from (163, 164).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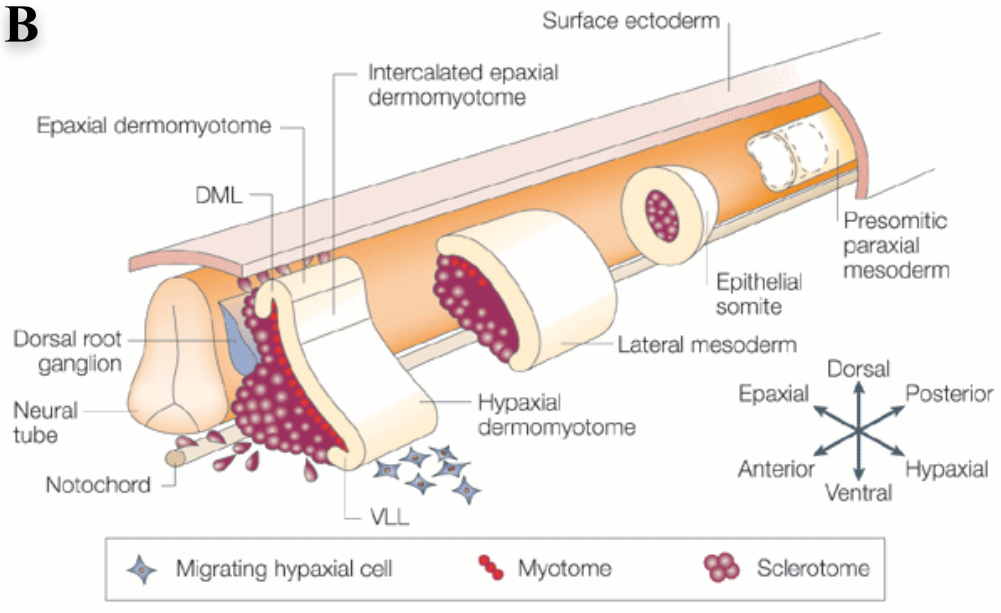
T= 1 cycle
T= 2 cycles
Somitogenesis
Determination front
Axis elogation
Retinoic acid
Mesp2
FGF/Wnt signaling
Cyclic Lfng


B
Surface ectoderm
Intercalated epaxial dermomyotome
Epaxial dermomyotome
DML
Presomitic paraxial mesoderm
Epithelial somite
Dorsal root ganglion
Lateral mesoderm
Neural tube
Hypaxial dermomyotome
Notochord
VLL
Dorsal
Epaxial
Posterior
Anterior
Hypaxial
Ventral
Migrating hypaxial cell
Myotome
Sclerotome

### 1.4.2 Differentiation of skeletal muscle *in vivo*

On a cellular level, the process of skeletal myogenesis can be broken down into three parts: i) the specification of mesodermal cells into pre-myogenic mesoderm; ii) the commitment of pre-myogenic mesoderm cells into myoblasts; and iii) the terminal differentiation of myoblasts into muscle (165). Skeletal myogenesis is controlled by a network of transcription factors and signalling molecules from neighboring tissues.

#### 1.4.2.1 Transcriptional regulation of skeletal myogenesis *in vivo*

Markers of early embryonic mesoderm include BraT and Mesp1 (166, 167). Pre-myogenic mesoderm expresses the transcription factors Pax3 and Pax7, which persist to mark satellite cells of the adult (168, 169). The Pax3/7 population appears to represent a progenitor pool that is maintained throughout embryogenesis and is responsible for almost all skeletal muscle (169, 170). Embryonic myoblasts express Myf5 (myogenic factor 5), MyoG (myogenin), MyoD (myogenic differentiation 1, MyoD1), and MRF4, which are the myogenic regulatory transcription factors (MRFs) that regulate skeletal muscle differentiation (reviewed in (171)). Embryonic myocytes express the proteins of the contraction-relaxation apparatus, including MHC and cardiac α-actin (172).

MyoD null mice do not exhibit gross muscle formation abnormalities, but have elevated Myf5 expression (173). Myf5/MRF4 null mice maintain the expression of MyoD and develop normal muscle (174). MyoD/Myf5/MRF4 null mice fail to express MyoG, develop muscle or myoblasts (175, 176) suggesting functional redundancy between these transcription factors. A conditional cell ablation approach using the expression of diphteria

toxin in Myf5-positive cells revealed an existence of Myf5-dependent and Myf5-independent myoblast lineages (177, 178). When Myf5-dependent lineage is ablated, MyoD-expressing myoblasts are expanded, thus rescuing the skeletal muscle phenotype (177, 178). These findings emphasize a significant heterogeneity and adaptability of the embryonic skeletal myogenic program.

The activity and expression of MRF proteins is modulated by a family of MEF2 transcription factors (reviewed in (179)). In *Drosophila*, expression of single MEF2 in epidermis can induce body-wall muscle development as evidenced by induction of expression of several muscle genes (180), whereas mutant MEF2 protein results in inhibition of muscle cell differentiation (181). In vertebrates, there are four MEF2 family members, MEF2 A, B, C and D. MEF2C is the first MEF2 member to be expressed in developing skeletal muscle at E8.5, followed by expression of MEF2A and MEF2D at E9.5 (182). Conditional knockout of MEF2C in skeletal muscle results in disorganized sarcomeres (183). This phenotype is similar to zebrafish studies, where knockdown of both MEF2C and MEF2D blocks sarcomere assembly and muscle function, but does not interfere with muscle fibre differentiation or fusion (184). In contrast, knockout of MEF2A, B or D proteins has little or no effect on murine skeletal muscle development (reviewed in (179)).

Contrary to studies in *Drosophila* (180), expression of constitutively active MEF2C in mice under the regulation of MyoG promoter and MEF2C enhancer does not lead to enhanced muscle differentiation, but rather drives fast to slow muscle fiber transformation (185). This correlates with inability of MEF2 proteins to convert non-muscle cells like fibroblasts into muscle, however, MEF2 proteins synergize with MRFs to enhance MRF-driven myogenic conversion of fibroblasts (186). Apart from enhancing MRF protein

activity, MEF2 transcription factors regulate the expression of MRF genes (187, 188). The expression of MEF2C, in turn, is regulated by MRF and MEF2 proteins during skeletal muscle development (189). Thus, MEF2 and MRF proteins create a positive feedback loop to maintain each other's expression during skeletal muscle formation.

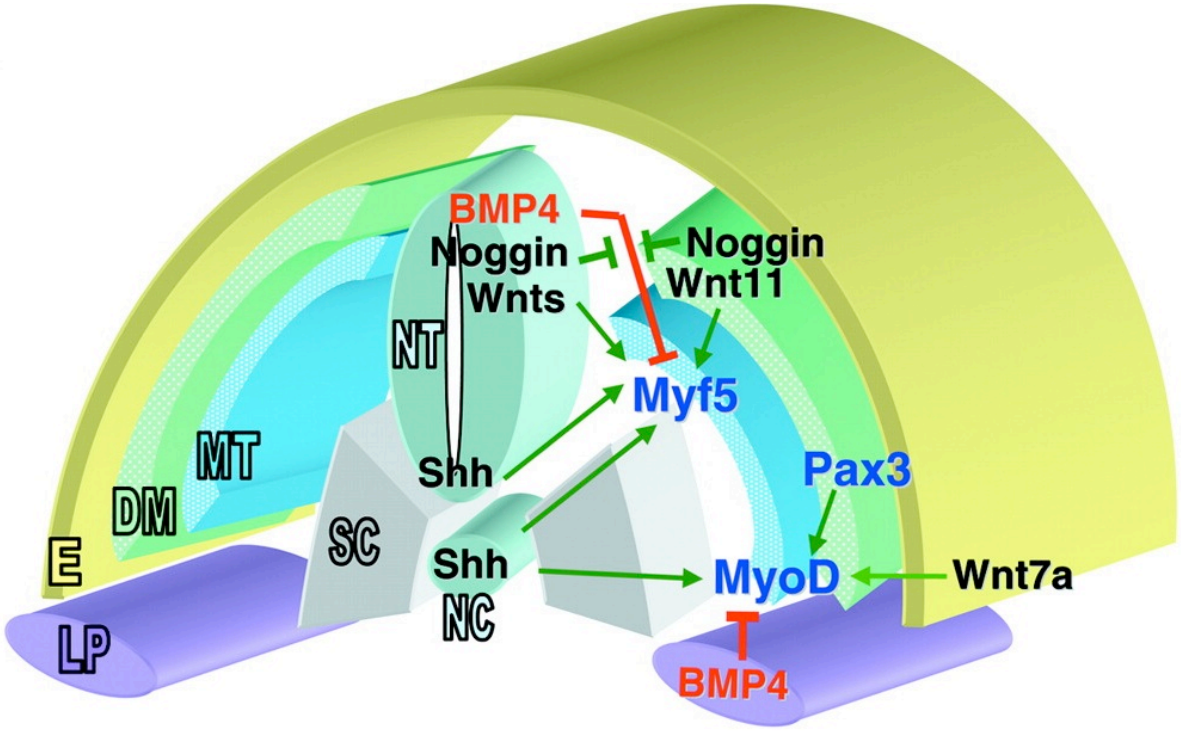
The expression of MRFs is also regulated by the upstream transcription factors, including Pax and Meox protein families. Although $Pax3^{-/-}$ mice lack the expression of Myf5, MyoD and MRF4 in hypaxial myotome (190), to date, only Myf5 is known to be directly regulated by Pax3 during hypaxial muscle development (191). While Pax7 is dispensable for embryonic muscle development (192), it is expressed in the somite and can replace Pax3 function (193). $Pax3^{-/-}/Pax7^{-/-}$ mice exhibit initial skeletal myogenesis, however, as myogenesis proceeds, severe cell death leads to almost a complete loss of skeletal myofibers by E13.5 (194). The expression of MyoD is dependent on Pax3, Myf5 and MRF4 (195), as Pax3/Myf5/MRF4 null mice lack trunk muscles and MyoD expression (195). Meox1 and Meox2 (mesenchyme homeobox 1 and 2) are expressed in the early somite (196) and are known to interact with members of the Pax family (197). Meox2 null mice exhibit downregulation of Pax3 and Myf5 expression and decrease in limb muscle differentiation (198). $Meox1^{-/-}/Meox2^{-/-}$ mice, however, exhibit more profound defects in muscle formation including a complete loss of Pax3 and severely reduced Myf5 expression as well as a severe disruption of somitogenesis leading to loss of somite derivatives (198).

#### 1.4.2.2 The role of signalling pathways during skeletal myogenesis *in vivo*

Skeletal myogenesis *in vivo* is controlled by multiple signalling pathways, including Shh, Wnt and BMP (Fig. 1.6) (reviewed in (199)).

**Figure 1.6. Signalling pathways regulate embryonic skeletal muscle formation.**

Dermomyotomal (DM, green) cells receive signals from surrounding tissues to induce (Wnts, Shh, Noggin) or inhibit (BMP4) commitment to myogenic lineage and expression of MRFs. Shh is expressed in notochord (NC, aquamarine) and is present in ventral neural tube (NT, aquamarine). Wnts are secreted from dorsal NT and surface ectoderm (E, yellow). BMP4 is secreted from dorsal NT and lateral plate (LP, purple) mesoderm and can be inhibited by Noggin. Wnt-11 expression in DM lip allows DM cells to involute into the myotome (MT, blue), a source of skeletal musculature. SC, sclerotome (grey). Adapted from (200), no permission is required.


BMP4
Noggin
Wnts
NT
Noggin
Wnt11
Myf5
MT
DM
E
LP
SC
Shh
NC
Shh
Pax3
MyoD
Wnt7a
BMP4

Several Wnts regulate somite patterning, including Wnts 1 and 3, which are secreted by dorsal neural tube and activate the canonical Wnt/β-catenin pathway, and Wnts 4, 6 and 7a, which are secreted by surface ectoderm and activate the non-canonical Wnt pathway (201). Wnt1 represses sclerotomal markers and expands the dermomyotomal marker Pax3 in chick (202), whereas mice lacking Wnt1 or Wnt3, partially lack dermomyotome and have reduced Pax3 and Myf5 expression (203). In accordance, dominant-negative ß-catenin/Engrailed (ß-cat/En) fusion protein disrupts somite structures and downregulates dorsal markers in *Xenopus* embryos (204). In pre-myogenic (paraxial) mesoderm explants, Wnt1 activates Myf5 expression, whereas MyoD is induced by Wnt7a and Wnt 6 (205) and its expression appears to be dependent on the non-canonical Wnt/PKC pathway (206).

Although β-catenin does not modulate the expresion of Shh (202), it activates the expression of Gli2 and Gli3, members of the Shh signalling pathway (207) during skeletal myogenesis *in vivo*. Gli2 is expressed in the pre-myogenic mesoderm and the dorsal part of developing somites (196, 208-210). It directly regulates the epaxial expression of Myf5 alone (211) and in synergistic cooperation with Wnt signalling pathway members (212). $Gli2^{-/-}$ /$Gli3^{-/-}$ mice exhibit a delay in epaxial dermomyotome formation and in Myf5 expression (209). In agreement, Shh null mice demonstrate a loss of epaxial dermomyotome (78, 209) and reduced activation of Myf5 gene (209), similar to Smo null mice (146). The expression of MyoD is also severely reduced in Shh null mice (213) and in chick embryos lacking notochord, a main source of Shh ligand (210). Furthermore, Shh activates MyoD expression in newly developing somites (210, 214). While the Shh signalling pathway has inductive roles during the formation of epaxial myotome (210, 213), it is important for the proliferation and survival of hypaxial myotome (215-217). More specifically, the exogenous Shh

maintains the expression of Myf5 and MyoD in mouse limb bud explants (216) and chick limb bud mesenchyme *in vivo* (215, 217), while mutant Gli1 downregulates MyoD expression in chick limb buds (217). Notably, inhibition of Shh signalling in developing zebrafish increases the number of $Pax3^{+}/Pax7^{+}$ muscle precursor cells in the somite and prevents further myogenic lineage differentiation (218, 219). Therefore, Hh signalling seems to be important for the formation of Myf5/MyoD-positive myotomal cells that have downregulated Pax3/7 expression and it appears to directly regulate the expression of at least one MRF member, Myf5. The molecular mechanism regulating MyoD expression by Shh/Gli2 remains to be determined.

While Shh and Wnt signalling pathways function in a positive manner to regulate skeletal muscle differentiation, BMP signalling pathways function to repress it. Overexpression of BMP upregulates Pax3 and downregulates MyoD expression, whereas inhibition of BMP signalling blocks Pax3 and activates MyoD expression in chick (220), similar to the role of BMP4 in murine lateral mesoderm (221). Noggin knockout mice, where negative regulation of BMP signalling is removed, show impaired myotome formation and high Pax3 expression (222). Thus, BMP signalling functions to expand the pool of $Pax3^{+}/Pax7^{+}$ muscle precursors, whereas Shh and Wnt signalling antagonize BMP signalling by activating Noggin expression (220, 223) to initiate their differentiation into Myf5/MyoD-positive myoblasts and the formation of myotome (reviewed in (199)).

### 1.4.3 Regulation of skeletal myogenesis in embryonic stem and embryonal carcinoma cells

Skeletal myogenesis in ES and EC cells appears to reflect the same temporal pattern of gene expression found during myogenesis in the embryo. Genes expressed in the primitive

streak during mesoderm induction, such as BraT, Wnt3a, and Wnt5b, are expressed immediately after mES and P19 EB formation (27, 224-226). Subsequently, the transcription factors expressed in the early somite, such as Pax3/7, Meox1/2 and Gli2, are expressed in mES, hES and P19 cells (13, 226-230). Finally, MEF2C and MRFs are expressed (11, 13, 28, 230, 231), marking the commitment to the skeletal muscle lineage.

One of the first gain-of-function studies in P19 and mES cells was performed using overexpression of MyoD (232, 233). Whereas MyoD can convert mouse embryonic fibroblasts into skeletal muscle (126), it converts pluripotent P19 monolayer cells into skeletal myocytes by only 3%, whereas aggregation of P19 cells stably overexpressing MyoD in the absence of DMSO results in 30% induction of P19 cell skeletal myogenesis (232), similar to mES cells (233). Thus, the differentiation of P19 EC and mES cells requires inductive events present in cellular aggregation. Indeed, almost all myogenic factors studied to date require cellular aggregation to induce the myogenic program in P19 EC cells (125, 151-153, 226-228, 232, 234-238).

Expression of dominant-negative MyoD/EnR repressive fusion protein, where MyoD activation domain is replaced with the mouse engrailed-2 repression domain, in P19 cells leads to reduced expression levels of Gli2 and Pax3 and ablated Meox1 and MRF expression and subsequently, failed skeletal myogenesis (238). The EnR repression domain works via histone acetyltransferase-dependent and -independent mechanisms, preventing rescue from other members of the MRF family. Thus, due to the dominant-negative nature of MyoD/EnR protein, it overrides wild type (wt) MRFs, and this result is in accordance with MyoD/Myf5/MRF4 null mice, which lack skeletal muscle (175, 176).

Wnt3a, activated ß-catenin (235), Pax3 (227) and Gli2 (236) induce skeletal myogenesis in P19 cells aggregated in the absence of DMSO, by upregulating pre-myogenic

mesoderm factors and the MRFs supporting their transcriptional activation function in skeletal myogenesis. The inductive properties of Pax3/7 are also shown in mES cells, where expression of exogenous Pax3 or Pax7 results in enhancement of MRF expression and skeletal muscle differentiation (239, 240). This is similar to the activation of MyoD by expression of active Pax3-FKHR protein in the mouse embryo (241). Dominant-negative fusion proteins of ß-catenin (235), Pax3 (227), Gli2 and Meox1 (236) inhibit skeletal myogenesis in the presence of DMSO at unique steps in the pathway. Expression of ß-cat/EnR in P19 cellular aggregation disrupts skeletal myogenesis by decreasing the expression of several primitive streak markers and abolishing expression of Gli2, Meox1, Pax3 and MRFs (235). Meox/EnR, Pax/EnR and Gli/EnR exert their effects after formation of mesoderm (227, 236). Whereas expression of Meox/EnR results in delayed expression of Gli2, as well as ablation of Pax3, Six1 and MRFs (236), expression of Pax/EnR shows normal expression of Gli2, but decreased expression of Meox1, Six1 and Eya2 and complete absence of MRFs (227). P19 EC stable cell lines overexpressing Gli/EnR demonstrate decreased expression levels of Meox1 and Pax3, which mark myogenic progenitors, and the absence of MRFs leading to ablation of skeletal myogenesis (236).

Thus, Wnt/β-catenin signalling functions at the stage of mesoderm/pre-myogenic mesoderm formation in pluripotent EC cells (235). Shh signalling functions at different stages of skeletal myogenesis in stem cells. First, Shh signalling affects the formation of myogenic progenitors, as evidenced by the induction or downregulation of Pax3 and Meox1 expression in P19 cells overexpressing Gli2 or Gli/EnR, respectively (236). Second, Shh signalling functions at the stage of committed skeletal myoblasts, as evidenced by the repression of MRF upregulation in P19 cells expressing Gli/EnR protein in the presence of DMSO (236). Although Gli2 is known to directly regulate the expression of Myf5 *in vivo*

(211), the ability of Gli factors to directly regulate the expression of other MRFs and to modulate the transcriptional activity of MRFs is currently not known.

### 1.4.4 Muscle regeneration by satellite cells

Adult muscle stem (satellite) cells (SCs) were first discovered by electron microscopy as mononucleated cells associated with skeletal muscle fibers (242). SCs have self-renewal capacity and are multipotent as they can differentiate into muscle, bone and brown fat, derivatives of mesoderm (243). They are closely related to muscle progenitors in somites and are believed to originate from Pax3/Pax7-positive dermomyotomal cells (169, 194, 244). In mice, embryonic Pax7 is essential for generation of satellite cells as evidenced by the constitutive knockout of Pax7 (245), yet its postnatal function is only critical in the first three weeks of life (192), when SCs cease proliferating and become quiescent (199). All quiescent SCs express Pax7 (169, 246) and are indispensable for muscle regeneration (247, 248). The SC pool is heterogeneous, consisting of ~90% of the cells that have expressed Myf5 at some point during satellite cell genesis, and ~10% of the cells that have never expressed Myf5 (249). The latter cells are more efficient at repopulating the satellite cell niche and can give rise to $Pax7^{+}/Myf5^{+}$ satellite cells during division (249).

Upon muscle injury, SCs divide and their progeny becomes activated by expressing Pax7, MyoD and Myf5 (reviewed in (250)). Whereas SCs isolated from $MyoD^{-/-}$ mice (251) exhibit delayed differentiation (252, 253) and enhanced engraftment into muscle (254), SCs isolated from $Myf5^{-/-}$ mice exhibit reduced amplification and precocious differentiation (255, 256). Thus, MyoD is important for the differentiation potential of myoblasts, while Myf5 is a critical regulator of satellite cell proliferation. The role of Myf5, however, is complicated by the analysis of SCs heterozygous for Myf5 expression or lacking Myf5 expression entirely,

as they are better at repopulating the satellite cell niche compared to their wild type counterparts (257). The latter study suggests that different levels of Myf5 can exhibit dual roles in the SC fate by maintaining the balance between SC self renewal and commitment (257). The proliferation of SCs is also maintained by Pax3/7 proteins. Pax7 negatively regulates the expression and activity of MyoD protein (258), and expression of Pax7 or degradation-deficient Pax3 proteins in myoblasts inhibits their myogenic differentiation (259, 260).

For the myoblast differentiation to occur, the expression of Pax3/7 needs to be downregulated, whereas the expression of MyoG and MRF4 needs to be upregulated (reviewed in (250, 261)). While MyoG and MRF4 do not play a role in SC development or maintenance (262), MyoG is known to negatively regulate Pax7 expression (263) and is important for the subsequent formation of myotubes, a critical event in muscle regeneration (reviewed in (261)).

The role of MEF2 factors appears to be important during SC activation, differentiation and fusion (231, 264). The inhibition of skeletal MLCK (myosin light chain kinase) signalling pathway, which regulates the activity of MEF2C protein, leads to the downregulation of MyoD and MyoG expression, indicative of reduced SC activation and differentiation (231, 265). Moreover, differentiation of primary myoblasts into myocytes and their fusion *in vitro* is impaired when both MEF2A and MEF2C are knocked down (264).

Muscle regeneration is also regulated by various signalling pathways. Insulin-like growth factor 1 (IGF-1) is known to enhance muscle regeneration, most probably by stimulating the proliferation and differentiation of SCs. Hepatocyte growth factor (HGF), on the other hand, slows down the muscle regeneration by stimulating the proliferation and inhibiting the differentiation of myoblasts. FGF signalling through FGF-2 and FGF-6 is

important for muscle regeneration by playing a positive role in SC proliferation (reviewed in (266, 267)). Non-canonical Wnt signalling regulates muscle regeneration through the expansion of $Pax7^{+}/Myf5^{-}$ SC pool by Wnt7a (268). Wnt7a-deficient mice exhibit reduced number of SCs following muscle injury, while ectopic Wnt7a enhances muscle regeneration (268). Interestingly, Notch signalling is important for SC proliferation, however, a switch to Wnt3a-mediated canonical Wnt signalling is important for myoblast differentiation and muscle regeneration (269). Shh signalling is also important for muscle regeneration by regulating the expression of Myf5 and MyoD during SC activation (270). Thus, signalling pathways are important for adult muscle regeneration at unique steps of the regeneration program.

The current problem of using satellite cells for muscle stem cell therapy lies in the irreversible upregulation of MRF expression upon SC isolation and propagation in tissue culture (reviewed in (199)). Importantly, SCs lacking the expression of MRFs can repopulate SC niche more efficiently than MRF-expressing SCs (254, 257, 271, 272). Thus, there is a need to develop SC culture methods that would increase SC renewal without the expression of MRFs to avoid SC activation and differentiation. To accomplish this, we need to better understand the molecular mechanisms regulating MRF expression during skeletal myogenesis in stem cells.

In summary, MRFs play a critical role during embryonic muscle formation and adult muscle regeneration (reviewed in (199)). The expression of MRFs is regulated by a number of transcription factors, like Pax3/7, Meox1/2 and MEF2 (177, 188, 196, 271) and signalling pathways, including Shh and Wnt (reviewed in (199)). The molecular mechanisms regulating the expression of MRFs, especially MyoD, by the signalling pathways, and the role of signalling pathways in the regulation of MRF protein activity are still poorly understood.

## 1.5 THESIS SUMMARY

### 1.5.1 Rationale

While it is clear that Hh signalling regulates a variety of developmental programs in the embryo, including neurogenesis, cardiac and skeletal myogenesis, the mechanism by which progenitor cells interpret Hh signals via Gli transcription factors, leading to specific cell types, is still poorly understood. The use of an *in vitro* stem cell model system allows for a detailed molecular analysis of pathways controlling differentiation. ES cells can give rise to the three embryonic germ layers, ectoderm, mesoderm or endoderm, using differentiation programs that closely resemble the hierarchy of transcriptional networks and signalling pathways of those observed in the embryo. Thus, ES cells are a paradigm of embryogenesis. Moreover, the pluripotency of stem cells makes them useful in various cell-based restoration therapies. However, before stem cells can be used in a therapeutic setting, we need to understand how to generate high yields of specific cell types. To do this, we need to better understand the molecular mechanisms driving various differentiation programs. Of the stem cell signalling molecules, Hh is an attractive subject to study as it controls differentiation processes during embryogenesis.

### 1.5.2 Hypothesis and objectives

I hypothesize that the extent of stem cell differentiation into cardiac and skeletal muscle, as well as neurons, is dependent on transcriptional regulation by the Hh signalling pathway.

The main focus of this project is the Gli2 transcription factor, a mediator of Hh signalling, which plays an important role during cardiac and skeletal myogenesis and

neurogenesis both *in vivo* and *in vitro*. To better understand how Gli2 participates in stem cell differentiation programs, I aimed to study the role of Gli2, its direct targets and interacting proteins in murine P19 embryonal carcinoma and adult muscle satellite cells. The objectives of my doctoral research are:

1. To study the transcriptional regulation of neurogenic bHLH factors by Gli2 during stem cell neurogenesis;
2. To study the relationship between Gli2 and MEF2C during cardiomyogenesis in stem cells;
3. To study the transcriptional regulation of myogenic bHLH factors by Hh signalling during skeletal myogenesis in stem cells.

### 1.5.3 Summary of results

In Chapter 2, experiments were designed to test the hypothesis that Gli2 directly regulates Ascl1/Mash1 expression during neurogenesis in P19 EC cells. We show that overexpression of Gli2 induces formation of neurons, but not astrocytes, in P19 EC cells. The overexpression of dominant-negative Gli/EnR protein leads to delayed neurogenesis and reduced gliogenesis in P19 cells. Analysis of neuronal markers demonstrates that expression of Gli2 enhances the expression of neurogenic bHLH factors, with the longest upregulation effect on Mash1 expression, whereas Gli/EnR reduces the levels of neurogenic bHLH transcripts. Chromatin immunoprecipitation (ChIP) assay demonstrates that Gli2 associates with *Mash1* gene elements. Finally, Gli2 activates the *Mash1* promoter, whereas Gli/EnR blocks Gli2-mediated activation of the *Mash1* promoter, in reporter assays. These findings

demonstrate a novel mechanism of Gli2-induced neurogenesis and identify Mash1 as a novel direct target of the Gli2 transcription factor.

In Chapter 3, we tested the hypothesis that Gli2 and MEF2C are involved in a regulatory loop and participate in a protein complex during cardiomyogenesis in P19 cells. We demonstrate that overexpression of Gli2 enhances, whereas expression of Gli/EnR reduces, MEF2C mRNA and protein levels during cardiomyogenesis in P19 EC cells. Furthermore, overexpression of MEF2C upregulates, whereas expression of MEF2C/EnR in cardiac muscle lineage downregulates, Gli2 mRNA and protein levels. ChIP assay demonstrates that Gli2 and MEF2C associate with each other's gene elements. Finally, we show that Gli2 and MEF2C form a protein complex capable of synergistically activating cardiac muscle related promoters. These findings illustrate a novel mechanism of Gli2- and MEF2C-mediated cardiomyogenesis in P19 EC cells.

In Chapter 4, we sought to test the hypothesis that Hh signalling, via Gli2, directly regulates the expression of MyoD and modulates its transcriptional activity. We show that Gli2 associates with *MyoD* gene elements while enhancing skeletal myogenesis in P19 EC cells, and activates *MyoD* promoter in reporter assays. Gli/EnR fusion protein blocks upregulation of MyoD and ablates skeletal myogenesis in P19 cells, further supporting the identification of MyoD as a novel direct target of Gli2. In addition, the inhibition of Hh signalling during satellite cell activation and in proliferating myoblasts reduces the expression of MyoD and MEF2C. We also demonstrate that endogenous Hh signalling is important for MyoD transcriptional activity in myogenic conversion assays and that Gli2, MEF2C and MyoD form a protein complex, which enhances MyoD protein function on skeletal muscle related promoters.

### 1.5.4 Conclusions

Our results provide a link between the regulation of tissue-restricted factors like Mash1, MEF2C and MyoD, and a general signal-regulated Gli2 transcription factor. We provide novel mechanistic insights into the neurogenic, cardiogenic and myogenic properties of Gli2 *in vitro*, and offer novel plausible explanations for its *in vivo* functions. The results obtained during my doctoral research may also be important for the development of future stem cell therapy strategies.

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# CHAPTER 2

# ASCL1/MASH1 IS A NOVEL TARGET OF GLI2 DURING GLI2-INDUCED NEUROGENESIS IN P19 EC CELLS*

## 2.1 Abstract

The Sonic Hedgehog (Shh) signalling pathway is important for neurogenesis *in vivo*. Gli transcription factors, effector proteins of the Shh signalling pathway, have neurogenic properties *in vivo*, which are still poorly understood. To study the molecular basis of neurogenic properties of Gli2, we used a well-established embryonic stem cell model, the P19 embryonal carcinoma (EC) cell line, which can be induced to differentiate into neurons in the presence of retinoic acid (RA). We found that, in the absence of RA, overexpression of Gli2 induced P19 EC cells to differentiate into neurons, but not astrocytes during the first ten days of differentiation. To our knowledge, this is the first indication that the expression of Gli factors can convert EC cells into neurons. Furthermore, Gli2 upregulated expression of the neurogenic basic helix-loop-helix (bHLH) factors, such as NeuroD, Neurog1 and Ascl1/Mash1 in P19 EC cells. Using chromatin immunoprecipitation assays, we showed that Gli2 bound to multiple regulatory regions in the *Ascl1* gene, including promoter and enhancer regions during Gli2-induced neurogenesis. In addition, Gli2 activated the Ascl1/Mash1 promoter *in vitro*. Using the expression of a dominant-negative form of Gli2, fused to the Engrailed repression domain, we observed a reduction in gliogenesis and a significant downregulation of the bHLH factors Ascl1/Mash1, Neurog1 and NeuroD, leading

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to delayed neurogenesis in P19 EC cells, further supporting the hypothesis that Ascl1/Mash1 is a direct target of Gli2. In summary, Gli2 is sufficient to induce neurogenesis in P19 stem cells at least in part by directly upregulating Ascl1/Mash1. Our results provide mechanistic insight into the neurogenic properties of Gli2 *in vitro*, and offer novel plausible explanations for its *in vivo* neurogenic properties.

## 2.2 Introduction

Central nervous system (CNS) development is orchestrated by numerous signalling pathways, including the Shh signalling pathway, which in mammals is mediated by the transcription factors Gli 1, 2, and 3 (reviewed in (1-3)). During neurogenesis *in vivo*, Shh-mediated signalling in the notochord and floor plate is essential and sufficient for the specification of ventral cell types in CNS (4-9). Based on mammalian knockout (KO) experiments reviewed in (1), Gli1 is a transcriptional activator that is dependent on Gli2 and/or Gli3-mediated transcription (1). Gli2 is a primary mediator of Shh signalling and mainly functions as a transcriptional activator (1), however, it was shown to have repressor functions in CNS and skeletal muscle development (10, 11). Gli3 is mainly a transcriptional repressor (1), but it also has been shown to have activator functions in embryonic development (9-11).

Gli proteins are known to have individual as well as combinatorial functions (12). Although Gli1 KO mice do not exhibit any phenotype (13), zebrafish embryos lacking Gli1 show partial ventral CNS patterning defects (14). Mice lacking Gli3 protein function exhibit neural tube closure defects (15, 16). Dysregulation of Gli2 is lethal and causes complete loss of floor plate and reduction of V3 interneurons (17, 18). Complementary functions of Gli proteins are evidenced by their ability to rescue, at least in part, each other's KO phenotype

(9, 11, 13, 19). Moreover, Gli proteins were recently shown to cooperate during neurogenesis *in vivo*, creating a dynamic physical network (20). Thus, all Gli proteins participate in early CNS development; however, teasing out the specific roles for each Gli factor has been somewhat complicated.

All Gli proteins have neurogenic properties *in vivo* as demonstrated by several studies (9, 10, 20, 21). Xenopus embryos injected with Gli1, Gli2 or Gli3 showed concentration-dependent ectopic neurogenesis. Of the three family members, Gli2 had the strongest neurogenic properties (21). It was later found that Gli2 can induce formation of motor neurons while inhibiting floorplate and neural crest differentiation (10). In a recent study, Gli2, as well as other Gli factors, were shown to regulate the expression of some neurogenic basic helix-loop-helix (bHLH) genes such as Ncam, Neurog1 and NeuroD (20). This correlates with the expression profile of Gli proteins in animal cap and neural plate primordium, which precedes the expression of neurogenic bHLH genes (22). This expression pattern is also observed during neurogenesis *in vitro*, where expression of Gli transcription factors coincides with expression of Sox1/2 (23), followed by expression of NeuroD1 (referred to as NeuroD herein), Ascl1 (also known as Mash1) and culminating in NeuN and β-III tubulin (Tuj1) (24-26).

Ascl1 belongs to bHLH transcription factors of the achaete-scute family and is important for the successful differentiation of neural progenitors *in vivo* (27-30). Ascl1 has recently gained new attention as a master-regulator of neurogenesis *in vitro* (31). Ascl1 was shown to convert mouse embryonic and postnatal fibroblasts into induced neurons (31), complementing previously described induction of neurogenesis in P19 EC cells (32). Ascl1

has also been proposed to be a downstream target of Shh signalling in adult neural progenitor cells (33), although whether the effect is direct or indirect is unknown.

Although the neurogenic properties of Gli transcription factors in primary neurogenesis have been established (10, 20, 21), the mechanistic insight into how Gli factors regulate the expression of neurogenic bHLH genes, such as Ascl1, and induction of neurogenesis, remains unknown. Since Gli2 was shown to have the strongest neurogenic properties in *Xenopus* (21), we aimed to study the molecular mechanism of Gli2-induced neurogenesis in a well-established embryonic stem cell model, the P19 EC cell line. P19 EC cells are isolated from a teratocarcinoma created by the transplantation of E7.5 mouse embryo cells into the testes of a C3H/He mouse (34). P19 EC cells resemble mouse embryonic stem (mES) cells as they maintain a pluripotent, undifferentiated state when cultured, and can differentiate into three germ layers, ectoderm, endoderm and mesoderm upon addition of various chemical stimuli (34-36). When P19 EC embryoid bodies are treated with RA, they differentiate into neurons on day 6, and astrocytes on day 10 (35). Neurogenesis in P19 cells has been extensively studied (24, 37-40) and is similar to neurogenesis in mES cells (41, 42). In this study we have found that overexpression of Gli2 induced neurogenesis, but not gliogenesis, in P19 EC cells during the first ten days of differentiation. We also found that Gli2 induced the expression of neurogenic bHLH factors such as NeuroD, Neurog1 and Ascl1. Conversely, a repressive dominant-negative Gli2 factor resulted in decreased gliogenesis and downregulated expression of NeuroD, Neurog1 and Ascl1 leading to delayed neurogenesis in P19 EC cells. Finally, Gli2 was found to bind directly to Ascl1 gene regulatory elements during Gli2-induced neurogenesis in P19 EC cells

and was able to activate the Ascl1 promoter *in vitro*. Therefore, expression of Gli2 can convert EC cells into neurons at least in part through the direct upregulation of Ascl1.

## 2.3 Materials and Methods

### 2.3.1 P19 EC cell culture

P19 EC cells (ATCC, #CRL-1825) and P19 EC cells stably overexpressing either Gli2, a dominant negative fusion protein of Gli2 with the engrailed repression domain, or an empty vector, termed P19[Gli2], P19[Gli/EnR], or P19[Control], respectively, were described in (43). Cells were cultured as described previously (44) and differentiated in 1% DMSO (vehicle) (Sigma-Aldrich, Canada) with or without 0.5 or 1 μM RA (Sigma Aldrich, Canada) as in (45, 46). Briefly, cells were aggregated or cultured in monolayer in the presence of chemical stimuli at the density of 100,000 cells/ml. RA and/or DMSO was added for the first 4 days of aggregation or throughout monolayer differentiation. Media was changed every other day.

### 2.3.2 Immunofluorescence

Antigenic analysis of differentiated cells was performed using neurofilament 68- (NF68) (Sigma-Aldrich, Canada), Tuj1- (β III tubulin) (Research Diagnostics, MA) or glial fibrillary acidic protein- (GFAP) (Zymed Laboratories, CA) specific antibodies as described in (47-49). Cy3- or FITC-conjugated secondary antibodies (Jackson Immuno Research Laboratories, USA) were used for detection of indirect immunofluorescence. Briefly, cells were fixed using ice-cold methanol or 4 percent paraformaldehyde (PFA) (Fischer Scientific, Canada), and incubated with primary and secondary antibodies in phosphate buffer saline (PBS) with or without 3% BSA (Serologicals Proteins Inc, IL) and 0.3% Triton X-100 (Bio-

Rad Laboratories, Canada). Hoechst dye was used as a nuclear marker. Indirect immunofluorescence was captured using a Leica DMI6000B microscope (Leica Microsystems GmbH, Germany). Images were collected at 400x magnification using a Hamamatsu Orca AG camera (Hamamatsu Photonics, Germany) and processed using Velocity 4.3.2 software (Perkin Elmer, Canada).

### 2.3.3 Quantitative Polymerase Chain Reaction (QPCR) analysis

RNA from differentiating P19 EC cells was harvested using RNeasy Mini Kit (Qiagen, Canada) and analyzed using real-time quantitative PCR (QPCR) as described in (48, 50). Briefly, 1 μg of RNA was reverse-transcribed (RT) to synthesize cDNA using Quantitect Reverse Transcription Kit (Qiagen, Canada). One-twentieth of the RT reaction was used as a template for QPCR amplification using the specific primers listed in Appendix G and the FastStart SYBR Green kit (Roche Applied Sciences, Canada) or Promega GoTaq qPCR Master Mix (Promega, WI). Data was acquired using ABI7300 and ABI7500 QPCR (Applied Biosystems, CA) or Eppendorf Realplex2 (Eppendorf, Canada) instruments, normalized to β-actin and analyzed as described in (51). Data represents mean ± SEM from three independent biological experiments and using two clonal populations per cell line.

### 2.3.4 Chromatin immunoprecipitation (ChIP) analysis

150 μg of chromatin from day 4 differentiating P19[Gli2] cells in the absence of RA was immunoprecipitated using 2 μg of Gli2-specific (Santa Cruz, G-20) or goat IgG non-specific antibodies (Invitrogen, Canada) and analyzed as described in (52). Briefly, cells were cross-linked with 4 percent formaldehyde (Fischer Scientific, Canada) and chromatin was sheared as described in (52). Sheared chromatin was incubated with Gli2 or IgG

antibodies and the immune complexes were captured using protein G sepharose beads as described in (52). Gli2 or IgG-bound chromatin was quantified as a percent chromatin input using QPCR analysis as described above. Data represents mean ± SEM from three independent biological experiments. Primers listed in Appendix H were designed for specific conserved Gli binding motifs, which were identified as described in (53).

### 2.3.5 Immunoblot analysis

P19[Control] and P19[Gli2] cells were differentiated without RA as described above. On days 0, 4, 6 and 9 cells were washed twice with ice-cold PBS and lysed with RIPA buffer containing 1x protease inhibitor cocktail (Roche, Canada) and 0.5 mM phenylmethanesulfonylfluoride (PMSF) (Sigma-Aldrich, Canada). Lysates were clarified by centrifugation for 15 min at 13,000 g. 20 μg of total protein was resolved using 4-12% gradient NUPAGE gels (Invitrogen, Canada) according to the manufacturer's protocol using MOPS SDS running buffer. Resolved proteins were transferred to polyvinylidene fluoride (PVDF) or nitrocellulose membranes, blocked in 5% milk, and reacted with Gli2- (54), NF68- (Sigma-Aldrich, Canada), α-tubulin- (Sigma-Aldrich, Canada) or β-actin-specific antibodies (Sigma-Aldrich, Canada). Signal was detected using Horseradish Peroxidase (HRP)-conjugated secondary anti-mouse (Cell Signalling, MA) or anti-rabbit (Santa Cruz, CA) antibodies, followed by a chemiluminescence reaction using Pierce ECL substrate (Fisher Scientific, Canada).

### 2.3.6 *Ascl1* promoter analysis

HEK-293 cells were plated at a density of 300,000 cells per 35 mm tissue culture grade dish and transiently co-transfected 24 h later using FuGENE (Promega, WI) with a

total amount of 4 μg of DNA with or without Gli2 and/or Gli/EnR expression plasmid described in (43) and a luciferase expression vector driven by *Ascl1*-8 kb promoter (termed Ascl1-luc) described in (55). Transfection efficiency was monitored by transfecting Renilla as described in (52). 24 h after transfection, cells were washed twice with ice-cold PBS and lysed according to the Dual Luciferase Kit protocol (Promega, WI). Luciferase activity was assayed using 10-15 μl of lysate and LmaxII384 luminometer (Molecular Devices, USA).

### 2.3.7 Statistical analysis

ANOVA followed by post-hoc Tukey HSD test was performed using XLSTAT software (Addinsoft, NY) to determine statistical significance between mean values of two groups (*, $p<0.05$; **, $p<0.01$).

## 2.4 Results

### 2.4.1 Gli2 is expressed during neurogenesis in P19 EC cells.

We first sought to determine whether Gli2 is expressed during endogenous P19 EC neurogenesis. P19 EC cells were aggregated for 4 days in the presence of DMSO, with or without RA, and then plated into tissue culture dishes in the absence of drug. Cells were fixed on days 6 and 10 for examination by immunofluorescence. P19 cells were able to differentiate into Tuj1- and NF68- positive neurons by day 6 as well as GFAP-positive astrocytes by day 10 in the presence, but not in the absence, of RA (Fig. 2.1A - C), in accordance with previous reports (35).

Neurogenesis was also followed by QPCR analysis of the expression of several neurogenic markers as well as by the loss of embryonic stem cell pluripotency markers, Nanog and Sox2 (56, 57) (Fig. 2.1D) during a 10-day time course of P19 cell differentiation

with (+RA) and without RA (-RA). Expression of Nanog and Sox2 was downregulated by days 1-2 or 2-3 of differentiation + or –RA, respectively (Fig. 2.1D, panels Nanog and Sox2). Thus, P19 EC cells lost pluripotency markers during differentiation under both conditions. Furthermore, Sox2 is also a marker of neural progenitor cells *in vitro* (58) and Sox2 transcripts were detected on days 3-5 of differentiation +RA but not –RA, supporting the RA-induction of neural progenitors cells in these cultures (Fig. 2.1D, panel Sox2). Expression of Nestin, which is present in neural, glial and muscle progenitor cells (59, 60), was upregulated by day 3 or 5 of differentiation + or – RA, respectively (Fig. 2.1D, panel Nestin). Notably, MEF2C, which is expressed during P19 EC neurogenesis (61, 62), was upregulated on days 1-3 of differentiation +RA but not –RA (Fig. 2.1D, Panel MEF2C). Subsequent upregulation of MEF2C on days 4-10 could be indicative of cardiac or skeletal myogenesis in –RA differentiation (63). Expression of the neuronal bHLH factors, NeuroD, Ascl1, and Neurog1, peaked from days 3-5 of differentiation +RA but not - RA (Fig. 2.1D, panels NeuroD, Ascl1 and Neurog1). Therefore, neuronal markers were expressed during days 3-5 and their expression was specific to RA-induced differentiation of P19 cells.

**Figure 2.1. Induction of neurogenesis in P19 EC cells by RA.**

P19 cells were differentiated using embryoid bodies in the presence of RA as described in (35). **(A)**: Formation of Tuj1-, and NF68- positive cells with neuronal morphology on day 6 and GFAP-positive cells with astrocyte morphology on day 10 of RA-induced (+RA) differentiation. Nuclei were stained with Hoechst, scale bar is 30 μm. **(B)**: P19 EC cells fail to form Tuj1-, NF68- and GFAP- positive cells in the absence of RA (-RA) on the days indicated. Nuclei were stained with Hoechst, scale bar is 30 μm. **(C)**: Tuj1-, NF68- and GFAP-positive cells from (A-B) were counted in 10 random fields and expressed as % of the total number of nuclei (3,000 nuclei). **(D)**: The temporal pattern of expression of indicated genes during P19 EC differentiation +/-RA. Representative QPCR analysis is shown in which fold changes are relative to day 0. Error bars represent +/- SEM.

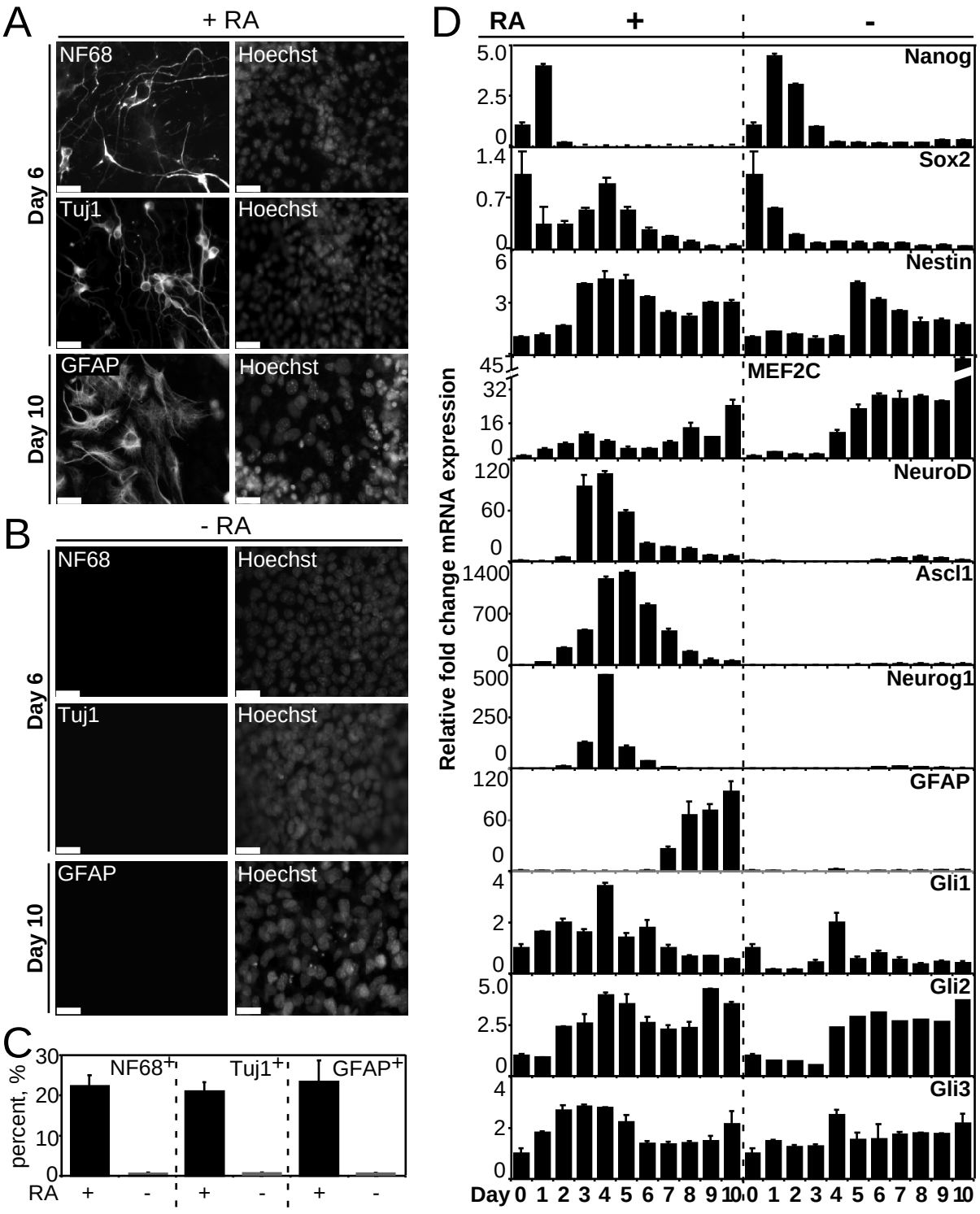

A
+ RA
NF68
Hoechst
Tuj1
Hoechst
GFAP
Hoechst
Day 6
Day 10
B
- RA
NF68
Hoechst
Tuj1
Hoechst
GFAP
Hoechst
Day 6
Day 10
C
percent, %
30
20
10
0
NF68+
Tuj1+
GFAP+
RA + - + - + -
D
RA + -
Nanog
Sox2
Nestin
MEF2C
NeuroD
Ascl1
Neurog1
GFAP
Gli1
Gli2
Gli3
Relative fold change mRNA expression
Day 0 1 2 3 4 5 6 7 8 9 10 0 1 2 3 4 5 6 7 8 9 10

**Table 2.1. Summary of gene expression for P19 cells treated + and - RA, P19[Gli2] cells treated - RA, and P19[Gli/EnR] cells treated + RA.**

| Cell line and treatment | Gli1 | Gli2 | Gli3 | Gli/ EnR | Nanog | Sox2 | Nestin | MEF2C | NeuroD | Ascl1 | Neurog1 | GFAP | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P19+RA | + | + | + | N/A | - | + | + | + | + | + | + | + | Fig. 2.1D |
| P19-RA | + | + | + | N/A | - | - | + | + | - | - | - | - | Fig. 2.1D |
| P19[Gli2]-RA | + | ++ | + | N/A | - | +/- | + | +# | + | + | + | +/- | Fig. 2.3 and Chapters 3-4 |
| P19[Gli/En] +RA | - | - | - | ++ | +/-* | - | - | + | - | - | - | - | Fig. 2.5 |



"++" means high upregulation as a result of overexpression, "+" means upregulation, "+/-" means no change, and "-" means downregulation of gene expression as compared to day 0. "N/A" means not applicable. For P19[Gli2] and P19[Gli/EnR] cell lines gene expression was compared to their respective control cell lines. * Expression of Nanog was downregulated only in undifferentiated P19[Gli/EnR] cells; # Chapters 2 and 3.

The expression of GFAP, a glial marker (35), was specific to RA-induced differentiation and was upregulated starting at day 7 (Fig. 2.1D, panel GFAP). Transcription factors Gli1-3 were expressed throughout the differentiation and were elevated during days 2-6 of RA-induced differentiation (Fig. 2.1D, panels Gli1, Gli2 and Gli3). Therefore, Gli factors, including Gli2, are expressed during P19 EC neurogenesis. The summary of gene expression from Fig. 2.1 is listed in Table 2.1.

### 2.4.2 Gli2 upregulates expression of neurogenic bHLH factors and induces neurogenesis in P19 EC cells

To test whether Gli2 has neurogenic properties in stem cells, we first aimed to establish a stem cell model, where parental differentiating stem cells would fail to undergo neurogenesis. If overexpression of Gli2 resulted in neurogenesis in the context of this model, it would indicate that Gli2 possessed neurogenic properties *in vitro*. Based on the results from Fig. 2.1, -RA differentiation was chosen to study the effect of Gli2 on neurogenesis.

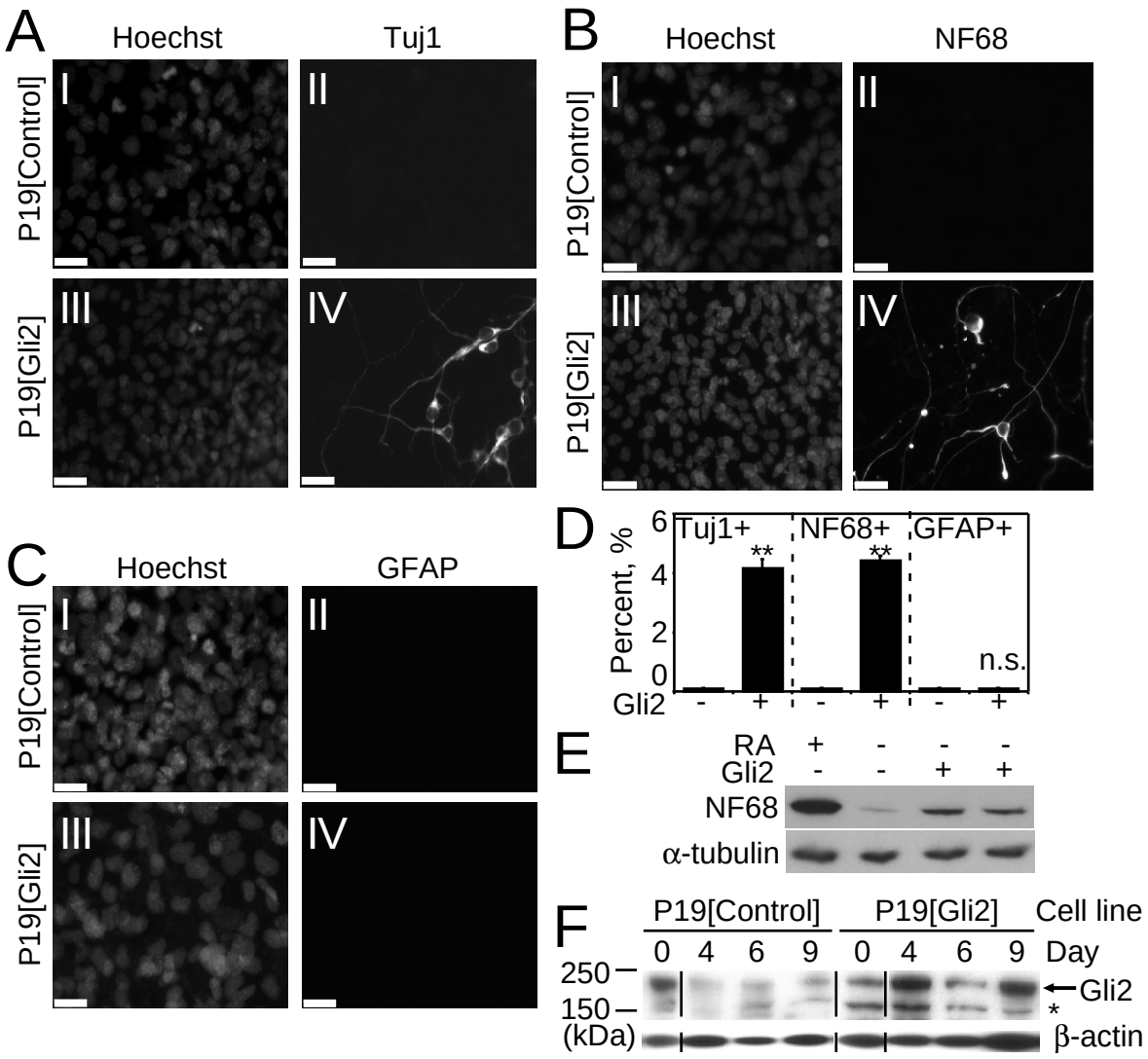
We differentiated P19 EC cells which stably overexpressed Gli2 (termed P19[Gli2]), in –RA conditions and examined for the presence of neurogenic markers by immunofluorescence and western blot analysis (Fig. 2.2). On day 6 of differentiation, Tuj1- and NF68-positive cells with neuronal morphology were seen in P19[Gli2] cultures, indicating that neurogenesis was indeed induced (Fig. 2.2A, panels III and IV and Fig. 2.2B, panels III and IV). P19[Control] cells failed to undergo neurogenesis under the same conditions (Fig. 2.2A, panels I and II and Fig. 2.2B, panels I and II). On day 10 of differentiation, the absence of GFAP-positive cells in both P19[Control] (Fig. 2.2C, panels I and II) and P19[Gli2] (Fig. 2.2C, panels III and IV) cells indicated no or delayed gliogenesis.

Thus, overexpression of Gli2 induced neurogenesis but not gliogenesis in aggregated P19 cells in the first ten days of differentiation.

To estimate the extent of neurogenesis induced by exogenous Gli2, Tuj1- and NF68-positive cells were counted and normalized to the number of Hoechst stained nuclei. P19[Gli2] cells differentiated into neurons by day 6, and they represented about 4 percent of total cells (Fig. 2.2D). No neurons were detected in P19[Control] cells differentiated under the same conditions (Fig. 2.2D). This result was confirmed by immunoblot analysis using NF68 antibodies, which showed an induction of NF68 protein in two clonal populations of P19[Gli2] cells when compared to the P19[Control] cell line (Fig. 2.2E). P19 EC cells differentiated in the presence of RA served as a positive control (Fig. 2.2E). Gli2 protein expression was confirmed by western blot analysis to be at higher levels in P19[Gli2] cells compared to control cells on days 4, 6, and 9, with the highest levels of Gli2 protein observed on day 4 (Fig. 2.2F). Thus, the expression of exogenous Gli2 in P19 EC cells leads to induction of neurogenesis, thereby confirming the neurogenic properties of Gli2 *in vitro*.

**Figure 2.2. Expression of Gli2 induces neurogenesis in P19 EC cells.**

**(A-C)**: P19[Gli2] and P19[Control] cells were stained with Tuj1 and NF68 antibodies on day 6 or GFAP antibodies on day 10 of –RA differentiation. Nuclei were stained with Hoechst, scale bar is 30 μm. **(D)**: Tuj1-, NF68- and GFAP-positive cells from (A)-(C) were counted in 10 random fields and expressed as a percentage of the total number of nuclei (10,000 cells; n=4) (**$p<0.01$, n.s. = not significant). **(E)**: NF68 immunoblot using total protein from day 6 differentiated P19, P19[Control] and two clonal populations of P19[Gli2] cell lines. P19 cells were differentiated in the presence of RA and served as a positive control. α-tubulin served as a loading control. **(F)**: Total protein from –RA differentiating P19[Control] and P19[Gli2] cells was harvested on the days indicated, separated and immunoblotted with Gli2-specific antibodies. β-actin served as a loading control. Asterisk denotes non-specific binding of Gli2 antibodies.

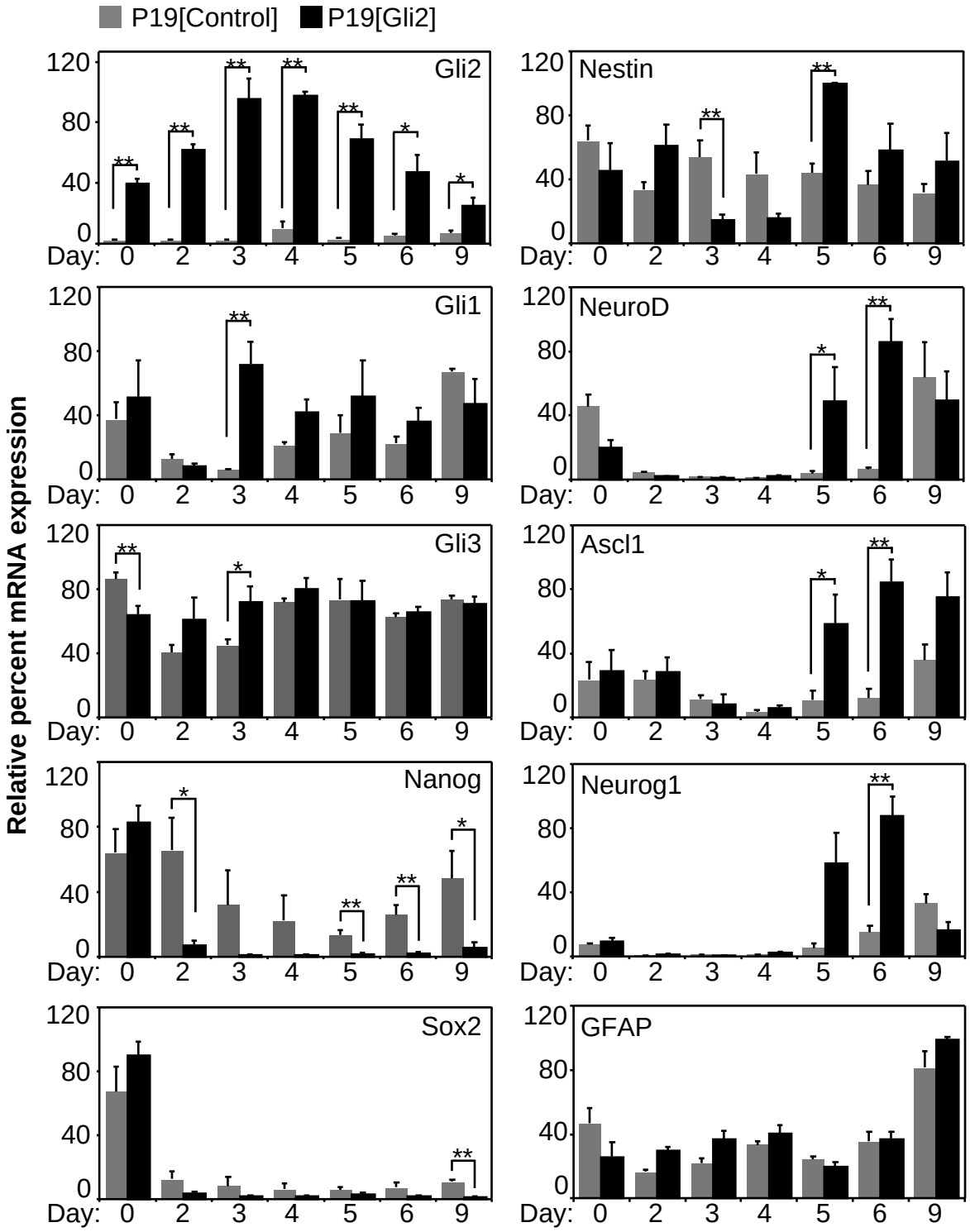

A
Hoechst
Tuj1
P19[Control]
P19[Gli2]
I
II
III
IV
B
Hoechst
NF68
C
Hoechst
GFAP
D
Tuj1+
NF68+
GFAP+
Percent, %
6
4
2
0
**
**
n.s.
Gli2
E
RA
Gli2
NF68
α-tubulin
F
P19[Control]
P19[Gli2]
Cell line
0 4 6 9 0 4 6 9 Day
250
150
(kDa)
Gli2
*
β-actin

To determine the expression pattern of neuronal markers induced by Gli2, we performed a time-course of QPCR gene expression analysis of markers from Fig. 2.1D. Overexpression of Gli2 was fairly stable throughout the differentiation (Fig. 2.3, panel Gli2). Upregulation of Gli1 and Gli3 expression on day 3 in P19[Gli2] cells as compared to the control cell line (Fig. 2.3, panels Gli1 and Gli3) suggested that overexpression of Gli2 activated the Shh signalling pathway. The expression of Nanog was significantly decreased in P19[Gli2] cells by day 2 (Fig. 2.3, panel Nanog), compared to P19[Control] cells, resembling the accelerated loss of Nanog observed during RA-induced differentiation (Fig. 2.1D). Sox2 was downregulated in both P19[Control] and P19[Gli2] cells by day 2 (Fig. 2.3, panel Sox2), indicating a loss of pluripotency.

Notably, later expression of Sox2 in P19[Gli2] and control cells remained low during most of the differentiation, suggesting that Gli2 did not induce neurogenesis via Sox2 upregulation (Fig. 2.3, panel Sox2). Furthermore, the expression of Nestin, which is expressed in neuro-glial and muscle progenitor cells (59, 60), was significantly downregulated on day 3, but upregulated on day 5 in P19[Gli2] cells (Fig. 2.3, panel Nestin). Finally, the expression of neurogenic bHLH factors NeuroD, Ascl1 and Neurog1 was upregulated by overexpression of Gli2 by days 5 or 6 (Fig. 2.3, panels NeuroD, Ascl1 and Neurog1). This correlated with the induction of neurogenesis as observed in Fig. 2.2. The expression of GFAP was not changed by overexpression of Gli2 even on day 9 of differentiation (Fig. 2.3, panel GFAP), which correlated with the absence of GFAP-positive cells on day 10 of differentiation (Fig. 2.2C).

**Figure 2.3. Expression of Gli2 induces expression of neuronal bHLH factors.**

Expression of indicated genes was assayed by QPCR analysis, n=4. RNA from differentiating P19[Control] (grey bars) and P19[Gli2] cells (black bars) was harvested on days 0, 2-6 and 9 of differentiation without RA. Error bars represent +/- SEM from at least two biological replicas using two clonal populations (*$p<0.05$, **$p<0.01$).


P19[Control]
P19[Gli2]
Relative percent mRNA expression
Gli2
Nestin
Gli1
NeuroD
Gli3
Ascl1
Nanog
Neurog1
Sox2
GFAP
120
80
40
0
Day: 0 2 3 4 5 6 9

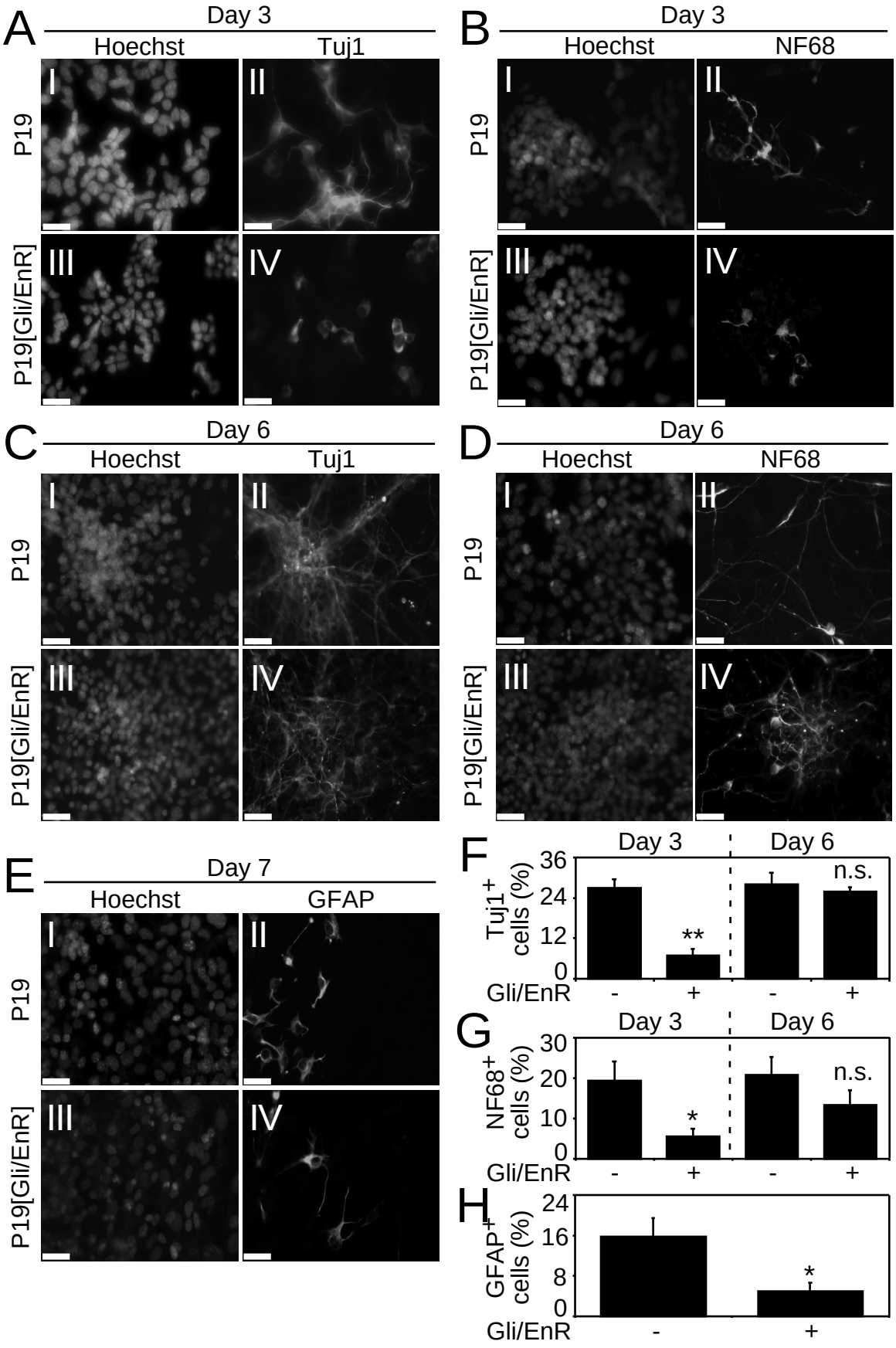
Gene expression analysis is summarized in Table 2.1. Thus, overexpression of Gli2 induced the expression of neuronal markers by days 5 and 6 of differentiation while gliogenesis was unaffected in the first ten days of differentiation.

### 2.4.3 Expression of dominant-negative Gli/EnR delays neurogenesis, reduces gliogenesis and reduces expression of neurogenic bHLH factors in P19 EC cells

Since Gli factors play complementary roles (12), we utilized a Gli2 dominant-negative construct, created by fusing the Gli2 DNA binding domain to the Engrailed repressor domain, termed Gli/EnR. Gli/EnR would bind to the Gli DNA binding domain and recruit repressors, inhibiting transcription in a fashion that cannot be rescued by other Gli factors, such as Gli1 or Gli3 (64, 65). Parental P19 and P19[Gli/EnR] cells were differentiated in the presence of RA using a monolayer procedure as described in (46), where the formation of neurons is detected within 3 days, and the formation of astrocytes is detected within 7 days. Antigenic analysis revealed a decrease in Tuj1- and NF68-positive neurons as compared to control P19 cells on day 3 of differentiation (Fig. 2.4A, 4B, 4F and 4G). However, by day 6, the levels of Tuj1- and NF68-positive neurons were similar (Fig. 2.4C, 4D, 4F and 4G), indicating that expression of Gli/EnR in P19 EC cells resulted in delayed neurogenesis. Day 7 differentiated P19[Gli/EnR] cultures showed a decrease in GFAP-positive astrocytes as compared to P19 control cells (Fig. 2.4E and 4H).

**Figure 2.4 Expression of Gli/EnR delays neurogenesis and decreases gliogenesis in P19 EC cells.**

Cells were differentiated using a monolayer procedure described in (46) in the presence of RA. **(A-D)**: Day 3 or day 6 differentiated P19[Gli/EnR] and P19 cells were stained with Tuj1- or NF68-specific antibodies. **(E)**: Day 7 differentiated P19[Gli/EnR] and P19 cells were stained with GFAP-specific antibodies. Nuclei were stained with Hoechst, scale bar is 30 μm. **(F-H)**: Tuj1-, NF68- and GFAP-positive cells from (A-E) were counted in 10 random fields and normalized with the number of nuclei (10,000 cells; n=4), *$p<0.05$, **$p<0.01$, n.s. = not significant.

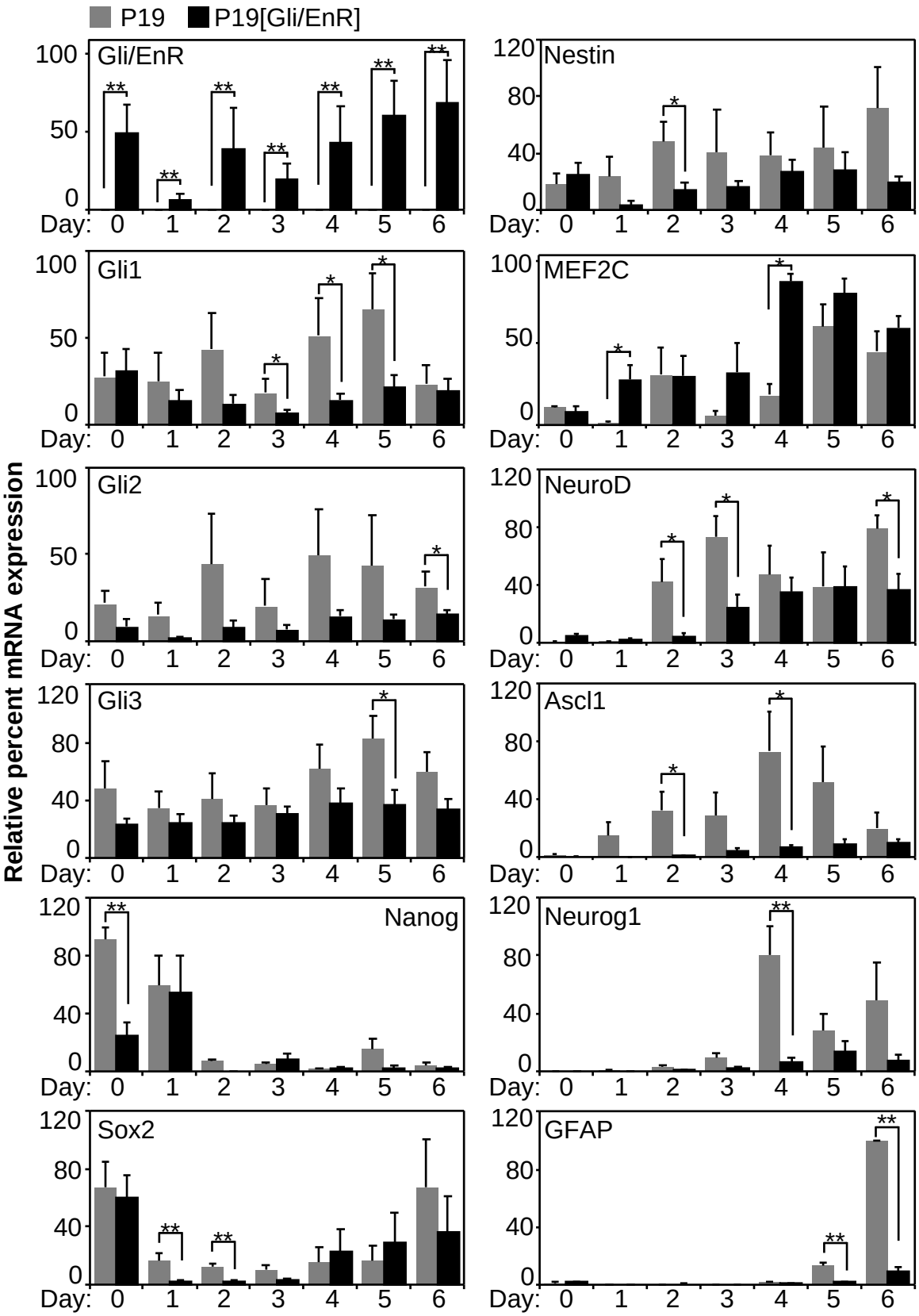

A
Day 3
Hoechst
Tuj1
P19
P19[Gli/EnR]
I
II
III
IV
B
Day 3
Hoechst
NF68
C
Day 6
Hoechst
Tuj1
D
Day 6
Hoechst
NF68
E
Day 7
Hoechst
GFAP
F
Day 3
Day 6
Tuj1+ cells (%)
36
24
12
0
**
n.s.
Gli/EnR
-
+
G
NF68+ cells (%)
30
20
10
0
*
H
GFAP+ cells (%)
24
16
8
0

To determine the expression pattern of neuronal markers affected by expression of Gli/EnR, we performed a time-course of QPCR gene expression analysis using markers from Fig. 2.1D. Gli/EnR was fairly stably overexpressed throughout the differentiation (Fig. 2.5, panel Gli/EnR). Downregulation of Gli1, Gli2 and Gli3 in P19[Gli/EnR] cells as compared to P19 control cells confirmed suppression of the Shh signalling pathway (Fig. 2.5, panels Gli1, Gli2 and Gli3).

The expression of Nanog, a direct target of Gli2 in neural stem cells (66), was significantly downregulated by the expression of Gli/EnR in undifferentiated cells (Fig. 2.5, panel Nanog, day 0), but was relatively unchanged by Gli/EnR expression during differentiation (Fig. 2.5, panel Nanog, days 1-6). Another direct target of Gli2 in neural stem cells, Sox2 (67), was expressed at the same level in undifferentiated P19[Gli/EnR] and P19 control cells (Fig. 2.5, panel Sox2, day 0). However, Sox2 was significantly downregulated by the expression of Gli/EnR on days 1 and 2 of differentiation (Fig. 2.5, panel Sox2). There was a trend in downregulation of the expression of Nestin in P19[Gli/EnR] cultures throughout the differentiation, however, the decrease in the Nestin mRNA levels was only statistically significant ($p<0.05$) on day 2 of differentiation (Fig. 2.5, panel Nestin). Thus, the neural progenitor markers Sox2 and Nestin were downregulated predominantly on days 1 and 2 of differentiation by dominant-negative Gli2 expression.

**Figure 2.5 Expression of Gli/EnR reduces expression of neuronal bHLH factors.**

Expression of indicated genes was assayed by QPCR analysis (Gli1, Gli3, n=8; Gli2, MEF2C, n=6; Gli/EnR, Sox2, Nestin, Ascl1, Neurog1, GFAP, n=4; NeuroD, n=3) by QPCR analysis. RNA from differentiating P19 (grey bars) and P19[Gli/EnR] cells (black bars) was harvested on days 0-6 +RA differentiation. Error bars represent +/- SEM from at least three biological replicas using two clonal populations (*$p<0.05$, **$p<0.01$).


P19
P19[Gli/EnR]
Relative percent mRNA expression
Gli/EnR
Nestin
Gli1
MEF2C
Gli2
NeuroD
Gli3
Ascl1
Nanog
Neurog1
Sox2
GFAP
Day: 0 1 2 3 4 5 6

Surprisingly, expression of MEF2C was upregulated on days 1 and 4 in P19[Gli/EnR] cells as compared to P19 control cells (Fig. 2.5, panel MEF2C). Since MEF2C can initiate neurogenesis and upregulate Ascl1 expression (47), it is possible that MEF2C may compensate, at least partially, for the Gli/EnR inhibition of neurogenesis.

The neurogenic bHLH factors, NeuroD, Ascl1, and Neurog1 were downregulated by the expression of Gli/EnR throughout the timecourse and were most significantly downregulated ranging from days 2-4 (Fig. 2.5, panels NeuroD, Ascl1, and Neurog1). By day 6 the extent of downregulation lessened with only NeuroD remaining significantly downregulated, suggesting a delay in neurogenesis rather than an inhibition, in agreement with the immunofluorescence analysis.

The expression of GFAP was severely downregulated in P19[Gli/EnR] cells on days 5 and 6 of differentiation (Fig. 2.5, panel GFAP), which correlated with a decrease in GFAP-positive cells in P19[Gli/EnR] cultures (Fig. 2.6H). Thus both the immunofluorescence and the gene expression analysis support an inhibition of gliogenesis by dominant negative Gli2 expression. The summary of gene expression from Fig. 2.5 is listed in Table 2.1.

In summary, a dominant negative Gli2 mutant attenuated neurogenesis in P19 EC cells shown by the downregulation of the neurogenic bHLH factors. In addition gliogenesis was inhibited, as shown by the downregulation of GFAP.

### 2.4.4 Gli2 binds to *Ascl1* gene regulatory elements and activates its promoter

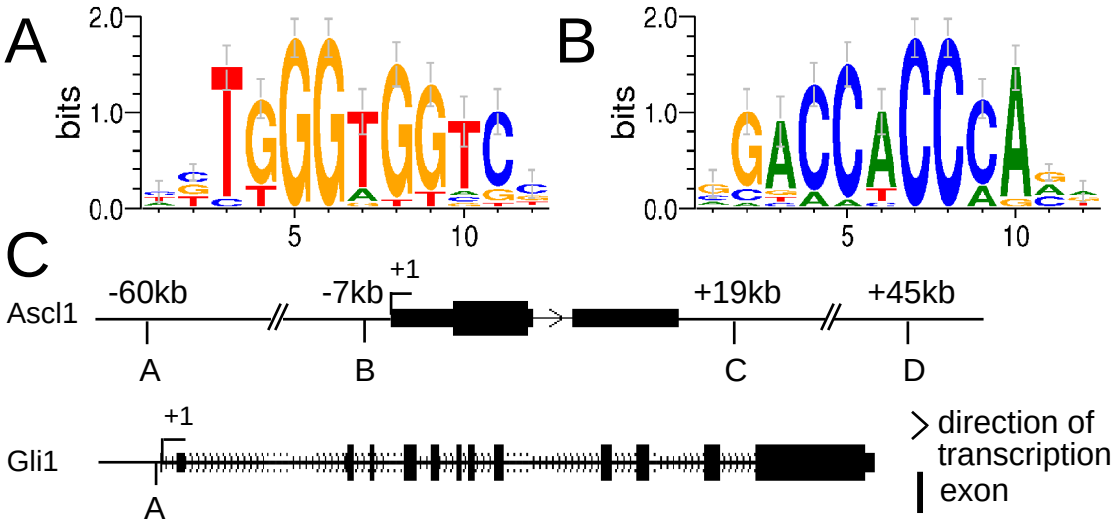
Since overexpression of Gli2 elevated the expression of several neurogenic *bHLH* genes, including *Ascl1*, (Fig. 2.3, panel Ascl1), which was previously proposed to be a downstream target of the Shh signalling pathway (33), we were interested to determine

whether Gli2 could bind directly to the *Ascl1* gene regulatory elements. *In silico* analysis of the *Ascl1* gene using the TRANSFAC Gli binding motif (Fig. 2.6A and 6B) revealed 4 theoretical, conserved Gli binding sites both upstream and downstream of the transcriptional start site (Fig. 2.6C and 6D), suggesting that Ascl1 might be a novel direct target of Gli2. Since day 4 differentiating P19[Gli2] cells showed the highest expression of Gli2 mRNA (Fig. 2.3, panel Gli2) and protein (Fig. 2.2F), this time point was chosen for ChIP analysis using Gli2-specific antibodies or IgG-nonspecific antibodies. We observed an enrichment of chromatin fragments corresponding to the *Ascl1* A-C sites, but not to the *Ascl1* D site (Fig. 2.6E) with Gli2 antibodies, as compared to non-specific IgG antibodies. The *Gli1* promoter was used as a positive control based on a previous report (68) (Fig. 2.6E). Thus, Gli2 binds directly to multiple sites located up- and downstream of the *Ascl1* gene.

To assess the functionality of the ChIP results, we performed *Ascl1* promoter analysis with Gli2. The *Ascl1* B site is located within the *Ascl1* promoter region, which has been characterized previously and contains 3 additional, non-conserved Gli binding sites (55). Promoter studies revealed that Gli2 directly activated the *Ascl1* promoter in a concentration-dependent manner up to 13 (± 1) fold (Fig. 2.7). Gli/EnR suppressed activation of the *Ascl1* promoter by Gli2, confirming the ability of Gli/EnR to bind to the Gli binding sequences and act as a repressor (Fig. 2.7). Thus, Gli2 elevates expression of Ascl1, binds directly to its gene regulatory regions and activates its promoter *in vitro*.

**Figure 2.6. Gli2 binds *Ascl1* gene regulatory elements in P19 EC cells.**

**(A-B)**: TRANSFAC (#M01037) Gli binding motif in forward and reverse direction, respectively. **(C)**: Custom tracks of *Ascl1* and *Gli1* genes using UCSC genome browser (http://genome.ucsc.edu). Triangles designate the direction of transcription, and black boxes designate exons. The *Ascl1* gene (+/- 100 kb) from mouse and human genomes was searched for conserved theoretical Gli binding as described in (53), which are designated as A-D. Their positions relative to the transcriptional start site (+1) are indicated as numbers. The known Gli binding site in the *Gli1* gene is designated as A (68) **(D)**: Comparison of mouse and human sequences of *Ascl1* A-D sites from (C). The sequence of the Gli binding site (GBS) is marked in bold. **(E)**: ChIP analysis showing enrichment by Gli2 antibodies of *Ascl1* chromatin fragments corresponding to sites A-C, from (C). Sheared chromatin from day 4 - RA differentiated P19[Gli2] cells was immunopurified using Gli2-specific (black bars) or IgG non-specific (grey bars) antibodies. The *Gli1* promoter served as a positive control. Percent chromatin input was calculated using QPCR analysis. Error bars represent +/- SEM from three biological replicas (*$p<0.05$, **$p<0.01$, n.s. = not significant).




D

| Ascl1 site | Species | Sequence | % GBS conservation |
|---|---|---|---|
| A | *H. sapiens* | ctc**ACTGGGTGGTCT**gca | 83.3 |
| | *M. musculus* | ctc**ACAGGGTGGTCA**gca | |
| B | *H. sapiens* | tcg**TTACCACCCACT**gca | 100 |
| | *M. musculus* | tca**TTACCACCCACT**gca | |
| C | *H. sapiens* | a-t**TTTGGGTGGGCT**gtg | 100 |
| | *M. musculus* | ggt**TTTGGGTGGGCT**gtg | |
| D | *H. sapiens* | ttc**TGTCAGTTGCCT**gtt | 66.7 |
| | *M. musculus* | atc**TGTTGGTGGTCT**ttt | |

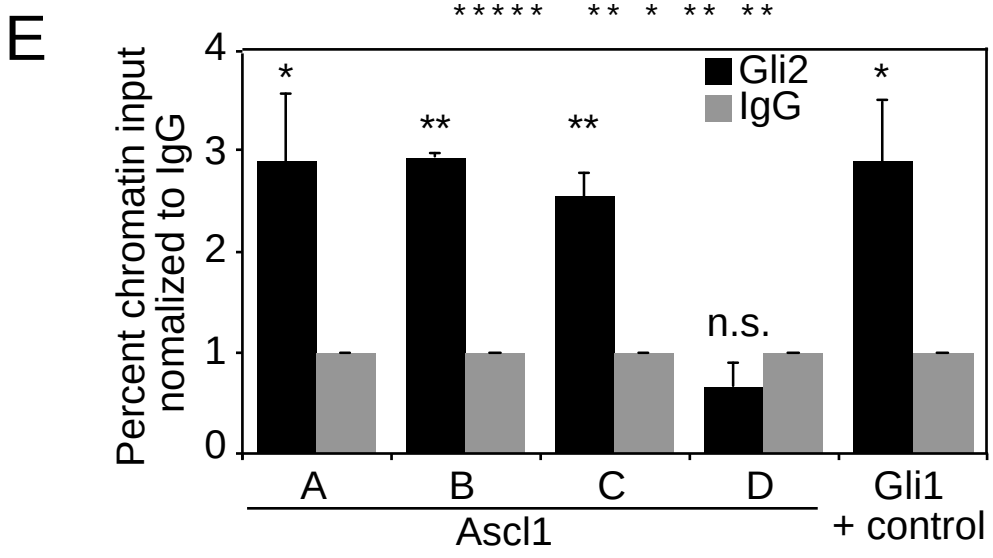
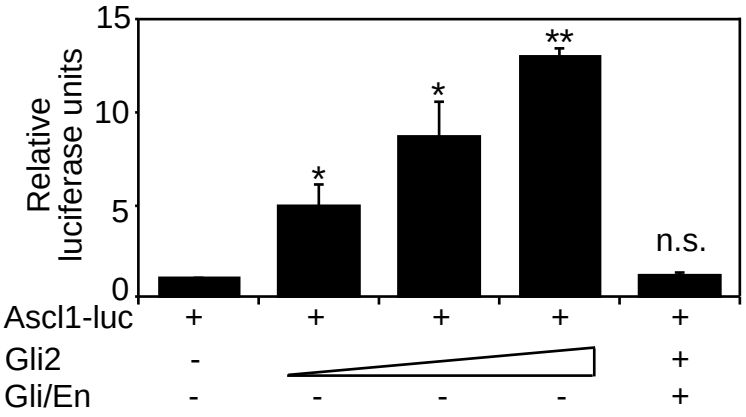


**Figure 2.7. Gli2 activates the *Ascl1* promoter.**

HEK-293 cells were transiently cotransfected with or without Gli2 and a construct containing the *Ascl1* 8 kb promoter driving the luciferase gene (Ascl1-luc) in ratios 2:1, 4:1 and 6:1 relative to Ascl1-luc. Equal parts of Gli/EnR were transfected together with Gli2 at a ratio of 4:1 relative to Ascl1-luc. Fold changes are relative to Ascl1-luc activity with the Ascl1-luc plasmid alone. Error bars represent +/- SEM from three biological replicas (*$p<0.05$, **$p<0.01$). No significant (n.s.) increase was observed in the presence of Gli/EnR.


15
10
5
0
Relative luciferase units
*
*
**
n.s.
Ascl1-luc + + + + +
Gli2 - +
Gli/En - - - - +

## 2.5 Discussion

In this chapter we have shown, for the first time, that overexpression of Gli2 induced neurogenesis, but not gliogenesis, in P19 EC cells during the first ten days of differentiation. We have also shown that Gli2 regulated the expression of neurogenic bHLH factors like NeuroD, Neurog1 and Ascl1 during Gli2-induced neurogenesis in P19 EC cells. The expression of repressive Gli/EnR resulted in a delay of P19 EC neurogenesis, as well as decrease in gliogenesis. The expression of neurogenic bHLH factors including Ascl1 was also decreased by the expression of Gli/EnR. Additionally, Gli2 directly bound to *Ascl1* gene regulatory elements during P19 EC Gli2-induced neurogenesis, and activated the *Ascl1* promoter *in vitro*. To our knowledge, this is the first indication that Gli factors can directly regulate neurogenic bHLH factor expression.

Our finding that Gli2 could induce neurogenesis supports and extends previous studies (9, 10, 20, 21). This is the first indication that expression of Gli2 induces, rather than enhances neurogenesis in an embryonic stem cell model. For example, other publications have demonstrated a 3-10 fold enhancement of neurogenesis by application of Shh agonist to mES (41) or by overexpression of Gli1 in hES cells (69). In our study, we observed an induction of neurogenesis from 0% of neurons in the control cell line to 4% in P19[Gli2] cells. Furthermore, the extent of induction of neurogenesis caused by expression of Gli2 in our study is similar to the extent of neurogenesis caused by other transcription factors (32, 47) and to that seen in mES and hES cells (41, 69). In contrast, mES and hES cells spontaneously differentiate into neurons (70, 71) and thus can only be used to study the enhancement of neurogenesis but not the induction by exogenous stimuli.

Our finding that expression of a dominant-negative Gli2 in P19 EC cells results in delayed neurogenesis supports and extends a previous study, where inhibition of the Shh signalling pathway in human ES cells resulted in reduced formation of Tuj1-positive neurons (72). Since the authors only tested one time-point for the presence of Tuj1-positive neurons, it is possible that a later time-point would reveal a restored amount of neurons in cultures treated with cyclopamine (72).

The expression patterns of Gli1-3 during P19 EC neurogenesis *in vitro* shown in this study is supported by previous work showing a role for Shh signalling in mES cell neurogenesis (23). Relatively low fold changes of upregulation for Gli2 (4 fold), as compared to Ascl1 (1400 fold) or NeuroD (120 fold), are due to high levels of Gli2 expression in undifferentiated cells (Fig. 2.2F) (73). Since Gli factors are expressed in multiple lineages, including myogenesis (43, 74) and neurogenesis (23), their expression is not specific to RA-induced neurogenesis (Fig. 2.1D). This is similar to expression of Nestin, which is present in both muscle and neuronal precursor cells (59, 60). Furthermore, a major effect of Shh signalling is the activation of Gli2 function, as opposed to the upregulation of Gli2 expression (75, 76).

It was previously shown that Gli proteins upregulated the expression of neurogenic bHLH factors such as Ncam, Neurog1 and NeuroD in *Xenopus* (20), however, the molecular mechanism for this phenomenon was not elucidated. Using P19[Gli2] cells, we were able to confirm the ability of Gli2 to elevate the expression of neurogenic bHLH factors (Fig. 2.3), although with a slight delay as compared to RA-induced expression of these factors (Fig. 2.1D). Moreover, the expression of dominant-negative Gli2 resulted in significant downregulation of NeuroD, Neurog1 and Ascl1 expression. This result correlates with a

previous report showing reduced expression of Ascl1 in neural progenitor cells treated with cyclopamine (33). In this study, Gli2 directly bound to the conserved Gli sites A-C in the *Ascl1* gene. The *Ascl1* promoter, which contains the B site, was activated by Gli2 in a concentration-dependent manner. The *Ascl1* C site falls within a novel highly conserved enhancer that directs expression in the neural tube, hindbrain and midbrain (77, element hs1114). The Gli proteins along with the Shh ligand are important in neural tube and brain development (78-80). *Ascl1* D site was the least conserved between mouse and human (Fig. 2.6B), and did not appear to be bound by Gli2. Therefore, Gli2 upregulates Ascl1 expression, binds to its gene regulatory elements and activates its promoter during Gli2-induced neurogenesis in P19 EC cells.

Notably, the *Ascl1* gene was not reported to be bound by Gli1 in a genome-wide ChIP-microarray analysis of mES cells undergoing neurogenesis (81), however its expression was reported to be attenuated by Shh in both mES (81) and adult neural stem cells (33). Furthermore, it is possible that *Ascl1* gene element(s) were bound by Gli1 in differentiating mES cells, but were not included in the results due to high false-discovery rate cutoff in reported ChIP-microarray analysis (81). Finally, it is possible that Ascl1 is a direct target of Gli2, and not Gli1, during neurogenesis *in vitro*.

While Nanog is a direct target of Gli2 in adult neural stem cells (66) we did not observe easily explained changes in Nanog expression in differentiating P19[Gli2] or P19[Gli/EnR] cells. Under pluripotent monolayer conditions, Gli/EnR inhibited Nanog expression (Fig. 2.5), but Gli2 did not change Nanog expression (Fig. 2.3). The latter phenomenon might be explained by the similar levels of Gli2 protein expression in pluripotent undifferentiated P19[Control] and P19[Gli2] cells (Fig. 2.2F, day 0). In contrast,

during differentiation, Gli2 enhanced Nanog downregulation, whereas Gli/EnR did not affect Nanog expression. These results are likely due to the difficulty of comparing results in postnatal rats (66) to an embryonic stem cell model (34), which is heterogeneous and encompasses different developmental stages, including pluripotent stem cells, neural progenitors, and neurons. Thus, while Gli2 function is important for maintaining stem cell Nanog expression, it cannot further enhance it. Pluripotency was maintained, despite the decrease in Nanog expression, in part because Sox2 was still expressed (Fig. 2.5). Further, P19[Gli/EnR] cells could still differentiate into cardiac muscle (Chapter 3) and neurons (Fig. 2.4).

Sox2 was also shown to be a direct target of Gli2 during differentiation of neural stem cells derived from E14.5 murine telencephalon (67). Although gain- or loss-of-function of Gli2 did not affect Sox2 expression in the pluripotent monolayer stem cell stage, loss of Gli2 function delayed Sox2 upregulation at the neural progenitor stage (Fig. 2.5). Notably, expression of Gli2 did not upregulate Sox2 or Nestin mRNA at the predicted progenitor stage, although Nestin was upregulated later, at the same time as the bHLH neurogenic genes (Fig. 2.3). It is possible that Gli2 upregulated the expression of other Sox factors, like Sox1 and Sox3, which exhibit redundant biological functions (82). These results suggest that Gli2 may bypass the progenitor stage and induce neurogenesis through upregulation of the bHLH neurogenic genes.

Surprisingly, the levels of MEF2C were upregulated in P19 cells overexpressing Gli/EnR. Since MEF2C was shown to initiate neurogenesis and drive Ascl1 expression (47), as well as have anti-apoptotic functions important for the survival of cells during neurogenesis (62, 83, 84), it is possible that MEF2C is able to compensate for the Gli/EnR

inhibition of neurogenesis. Notably, on day 4 when MEF2C is greatly upregulated by Gli/EnR, Ascl1 is downregulated (Fig. 2.5), suggesting that MEF2C cannot bypass the inhibition of Ascl1 by dominant negative Gli2 at this time point. Since Gli/EnR is an active dominant negative mutant, it is possible that MEF2C could compensate for the simple loss of Gli2 signalling if Gli2 was knocked-down or -out. The relatively mild phenotype of P19[Gli/EnR] cells is consistent with previous reports showing that Shh signalling is not essential for the neural tube development (1, 17, 18).

The overexpression of Gli2 did not result in the formation of astrocytes in the first ten days of differentiation, whereas P19[Gli/EnR] cells showed reduced gliogenesis. Previous reports have demonstrated increased astrocyte formation in hES cultures differentiated in the presence of cyclopamine (72). The discrepancy in results might be due to the dominant-negative repressive effect of Gli/EnR, which is capable of overriding the activity of Gli factors (Fig. 2.7). Cyclopamine, on the other hand, binds Smo, and thus prevents activation of Gli transcription factors by Shh (85). However, other signalling molecules have been implicated in the activation of Gli factors, such as TGFβ (86) and Wnt (87). Moreover, Zic factors have also been implicated in modulating the transcriptional activity of Gli factors as well as in binding Gli binding sites in the chromatin (88). It is possible that expression of Gli/EnR caused a delay in gliogenesis, similar to neurogenesis, however, this hypothesis was not tested. To our knowledge, this is the first indication that dominant-negative Gli/EnR causes a delay in neurogenesis and a decrease in gliogenesis in P19 stem cells.

In summary, our findings indicate that Gli2 has neurogenic properties *in vitro*. Gli2 is able to directly regulate expression of the neurogenic bHLH factor, Ascl1, and convert P19 EC cells into neurons, but not astrocytes in the first ten days of differentiation. Dominant-

negative Gli2 is able to suppress expression of neurogenic bHLH factors and delay neurogenesis. Gli2 is probably not a sole regulator of Ascl1 expression during neurogenesis, as there are several other proteins, including Notch1 (89), MEF2C (90) and Hes1 (55), which were shown to regulate Ascl1 expression. Our findings unravel new molecular mechanistic insight into the neurogenic properties of Gli2 *in vitro*, thus offering novel plausible explanations for Gli2 neurogenic properties *in vivo*.

## 2.6 Acknowledgments

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# CHAPTER 3

# GLI2 AND MEF2C ACTIVATE EACH OTHER'S EXPRESSION AND FUNCTION SYNERGISTICALLY DURING CARDIOMYOGENESIS IN VITRO [*]

## 3.1 Abstract

The transcription factors Gli2 (glioma associated factor 2), which is a transactivator of Sonic Hedgehog (Shh) signalling, and MEF2C (myocyte enhancer factor 2C) play important roles in the development of embryonic heart muscle and enhance cardiomyogenesis in stem cells. Although the physiological importance of Shh signalling and MEF2 factors in heart development is well known, the mechanistic understanding of their roles is unclear. Here, we demonstrate that Gli2 and MEF2C activated each other's expression while enhancing cardiomyogenesis in differentiating P19 EC cells. Furthermore, dominant-negative mutant proteins of either Gli2 or MEF2C repressed each other's expression, while impairing cardiomyogenesis in P19 EC cells. In addition, chromatin immunoprecipitation (ChIP) revealed association of Gli2 to the *Mef2c* gene, and of MEF2C to the *Gli2* gene in differentiating P19 cells. Finally, co-immunoprecipitation studies showed that Gli2 and MEF2C proteins formed a complex, capable of synergizing on cardiomyogenesis-related promoters containing both Gli and MEF2 binding elements. We

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propose a model whereby Gli2 and MEF2C bind each other's regulatory elements, activate each other's expression and form a protein complex, which synergistically activates transcription, enhancing cardiac muscle development. This model links Shh signalling to MEF2C function during cardiomyogenesis and offers mechanistic insight into their *in vivo* functions.

### 3.2 Introduction

The mammalian heart is the first organ to develop and is essential for life. Perturbations in cardiogenesis can lead to congenital heart disease, the most prevalent birth defect worldwide. Heart development *in vivo* starts with the formation of the cardiac crescent, where the first heart field progenitor cells fuse to form the linear heart tube and give rise to the left ventricle. Second heart field progenitor cells then migrate to form pharyngeal and splanchnic mesoderm, which will form the right ventricle and the outflow tract (1, 2). In order to properly define and maintain the cardiac identity, Shh signalling pathway members and MEF2 proteins are required as shown by various animal models ((3-10) and reviewed in (1, 2)).

In mammals, the Shh signal is transmitted into the cell by the patched1/smoothened (Ptch1/Smo) regulatory complex and is mediated by transcription factors Gli 1, 2, 3 (reviewed in (11, 12)), which bind the TGGGTGGTC DNA consensus sequence (13). Gli1 acts as a transcriptional activator, but is dependent on Gli2 and/or Gli3-mediated transcription. Gli2 is a primary mediator of Shh signalling and functions mainly as a transcriptional activator. Gli3 is a transcriptional repressor (11). Using genetic inducible fate mapping, members of the Shh signalling pathway were shown to be expressed in murine myocardial progenitor cells starting from embryonic day (E) 7.0-8.0 (3). The expression of

Gli1 in some atrial and ventricular myocytes was confirmed in another study when tamoxifen was administered to the R26RGli1-CreERT2 embryos at E6.5 (10). Thus, embryonic cardiomyocytes and/or cardiac progenitors were exposed to Shh signalling during development.

The Shh pathway participates in the establishment of a proper number of cardiac progenitor cells during early vertebrate heart development in zebrafish (3). Inhibition of the Shh signalling resulted in an early defect in myocardial progenitor specification leading to reduction of both ventricular and atrial cardiomyocytes (3). Additionally, activation of Shh signalling resulted in an increase of cardiomyocytes (3). The importance of the Shh signalling pathway in mammalian heart development was demonstrated by total and tissue-specific knockout studies. $Smo^{-/-}$ mice showed delayed formation of heart tube with delayed Nkx2-5 expression (4), whereas $Ptc1^{-/-}$ mice, where the negative regulation of Shh signalling was removed, demonstrated upregulated Nkx2-5 expression during heart development (4). Moreover, in $Shh^{-/-}$ mice there were atrial septal defects and aberrant development of the outflow tract (5). Additionally, $Gli2^{-/-}Gli3^{+/-}$ mice showed cardiac outflow tract anomalies (6, 14). Tissue-specific removal of the Shh signalling pathway members in murine second heart field demonstrated their role in atrioventricular septation and the development of the outflow tract (8-10). In addition, Shh signalling was found to be important in proliferation of second heart field progenitors in chicken embryos (7). Therefore, Shh signalling via Gli2 is important for embryonic heart development.

In addition to Gli transcription factors, cardiomyogenesis is also regulated by MEF2 family members. The four vertebrate MEF2 proteins, MEF2A, MEF2B, MEF2C and MEF2D belong to the MADS box family of transcription factors and bind A/T rich DNA

sequence (T/C)TA(A/T)4TA(G/A) (15). MEF2C is the first MEF2 family factor to be expressed in heart myocardium progenitors starting from E7.5 (16, 17).

Loss-of-function mutations in the single Mef2 gene in *Drosophila* lead to a block of the development of all muscle cell types during embryogenesis (18). In mammalian embryogenesis, however, the four MEF2 family members play redundant roles and can rescue the phenotype resulting from a specific Mef2 gene knockout (reviewed in (15)). For example, Mef2c-null mice undergo cardiomyogenesis but die from a failure to undergo heart looping morphogenesis and proper development of the right ventricle as well as the outflow tract (19, 20). Cardiomyogenesis might be occurring due to a 7-fold increase in MEF2B protein observed in Mef2c-null mice, which can partially compensate for the phenotype. Overlapping functions of MEF2C and MEF2B are also supported by the evidence of normal development of Mef2b-null mice (19). The possibility of the phenotype rescue by other MEF2 family members was eliminated by expression of a dominant-negative fusion protein, of MEF2C with the engrailed repression domain (MEF2C/EnR) under the control of the *Nkx2-5* enhancer, which resulted in a severe disruption of cardiomyogenesis in mice (21). Thus, both Shh signalling and MEF2 family proteins play an important role in cardiomyogenesis *in vivo*.

The results from *in vivo* experiments have largely been reproduced *in vitro*. First, members of the Hedgehog signalling pathway and MEF2 transcription factors are expressed during cardiomyogenesis in embryonic stem cells (22-25). During cardiomyogenesis in embryonic stem (ES) cells, committed cardiac progenitor cells express MEF2C and GATA-4 transcription factors after the formation of the mesodermal cells as characterized by the expression of BraT and Oct-4. Cardiac progenitor cells terminally differentiate into

cardiomyocytes and express contractile proteins such as myosin heavy chain (MHC) and cardiac Troponin T (22).

A similar pattern of gene expression to ES cells can be seen in studies using pluripotent P19 embryonal carcinoma (EC) cells (26-28). P19 EC cells were originally isolated from an induced teratocarcinoma of male mouse testes by injection of isolated mES cells (26). These cells can contribute to tissues in live-born chimeric mice (29) and can undergo controlled differentiation into cardiac and skeletal muscle cells (up to 15%) *in vitro* when treated with dimethylsulfoxide (DMSO) (27, 28, 30-34). We have previously shown that retinoic acid regulates skeletal myogenesis in P19 EC, mouse and human ES cells through upregulation of Pax3/7-positive progenitor cells (35, 36), similar to a role for retinoic acid and Pax3/7 progenitors during embryogenesis (37, 38). Moreover, Nkx-MEF2C/EnR was found to abrogate cardiac myogenesis both during murine embryogenesis and in P19 EC cells (21). Similarly, Nkx/EnR impaired cardiac muscle formation *in vivo* (39) and in P19 EC cells (40). More recently, we found that skeletal myosin light chain kinase (MLCK) is important for skeletal myogenesis in mES and P19 EC cells, as well as in the activation of satellite cells via interaction and subsequent phosphorylation of the MEF2C protein (25). For Gli2 and MEF2C expression, previous publications demonstrate that the members of the Shh signalling pathway and MEF2 family members are expressed during cardiomyogenesis in P19 EC cells (41, 42) and P19CL6 cells (43, 44) as well as in cardiomyocytes derived from mES cells stably expressing the neomycin-resistance gene under the regulation of the cardiac α-myosin heavy chain promoter (23). Therefore, P19 EC cells use similar pathways as those identified in the embryo, mouse and human ES cells and are a suitable model to study cardiac and skeletal myogenesis *in vitro*.

Gain-of-function experiments in P19 EC cells revealed that both Gli2 and MEF2C are able to induce cardiomyogenesis through the upregulation of Nkx2-5, GATA-4 and BMP-4 factors (41, 45). Inhibition of Shh signalling *in vitro* resulted in aberrant cardiomyogenesis in P19 and P19CL6 cells (42, 43). Surprisingly, the ubiquitous expression of MEF2C/EnR, driven by the pgk promoter, enhanced cardiomyogenesis in P19 cells, likely by binding and inhibiting class II HDACs, resulting in the recruitment of non-cardiac muscle cells into the lineage (46). However, consistent with the gain-of-function experiments, the cardiac-restricted expression of MEF2C/EnR, driven by an Nkx2-5 enhancer, ablated cardiomyogenesis in P19 EC cells (21), demonstrating that MEF2 factors, or genes regulated by MEF2, are essential for cardiomyogenesis. Therefore, Shh signalling members and MEF2 proteins are important for cardiomyogenesis in stem cells.

Although Gli2 and MEF2C activate myogenic differentiation programs in a similar fashion (41, 45, 47, 48), and their physiological role in heart development is known ((3-6, 18, 21) and reviewed in (15)), their detailed function and the mechanism of their regulation is unclear. In this paper, we examined the relationship between Gli2 and MEF2C during mouse ES and P19 EC cellular cardiomyogenesis. We have found that Gli2 and MEF2C regulate each other's expression and associate with their respective gene elements as well as form a protein complex capable of synergizing on gene promoters. We therefore link, for the first time, the Sonic Hedgehog signalling pathway to the function of MEF2C protein during cardiomyogenesis *in vitro*.

## 3.3 Methods

### 3.3.1 Transgenic mice

$Gli2^{+/-}$ heterozygous mice (obtained from Dr. A. Joyner, Sloan-Kettering Institute, New York, NY; (49)) were maintained on CD1 background. $Gli2^{-/-}$ homozygous mice were generated by crossing two $Gli2^{+/-}$ heterozygous mice.

### 3.3.2 mES cell culture

D3 mES cells (ATCC, # CRL-1934) were maintained as described in (25, 35). For differentiation, cells were aggregated in hanging drops containing 800 cells each for the first 2 days. After culturing aggregates in suspension for an additional 5 days, they were plated on tissue-culture grade adherent plates or 0.1%-gelatin covered coverslips for the remaining 2 days of the 9 day differentiation protocol.

### 3.3.3 P19 EC cell culture

P19 EC cells (ATCC, #CRL-1825) were cultured and differentiated in the presence of 1% DMSO as previously described (50). Briefly, cells were aggregated in the presence of DMSO for 4 days and formed aggregates were plated onto 0.1%-gelatin covered coverslips or adherent tissue-culture grade dishes. P19 EC cells overexpressing various factors including P19[Gli2], P19[MEF2C], P19[Control], P19[Gli/EnR], P19[MEF2C-TAP] and P19[TAP] cells were described elsewhere (25, 47). P19[Nkx-MEF2C/EnR] cells stably overexpressing MEF2C/EnR under the regulation of the *Nkx2-5* enhancer (within the region from -9435 to -7353 nt relative to *Nkx2-5* transcriptional start site) were described in (21).

### 3.3.4 Immunofluorescence

On day 6 of P19 EC and day 9 of mES cell differentiation, cultures were fixed and incubated with MF20 monoclonal antibody supernatant to detect expression of MHC as previously described (50). Cy3-conjugated secondary antibodies (Jackson Immuno Research Laboratories, USA) were used to detect immunofluorescence as described in (51). Hoechst dye was used as a nuclear marker. Indirect immunofluorescence was captured using a Leica DMI6000B microscope (Leica Microsystems GmbH, Germany) or a Zeiss Axioscope microscope (Carl Zeiss MicroImaging GmbH, Germany). Images were collected at 200x or 400x magnification using Hamamatsu Orca AG camera (Hamamatsu Photonics, Germany) or Sony 3CCD camera (Sony Corporation, Japan) and processed using Velocity 4.3.2 (Perkin Elmer, Canada) or Axiovision (Carl Zeiss MicroImaging GmbH, Germany) software.

### 3.3.5 Quantitative PCR analysis

Total RNA from differentiating mES and P19 EC cells was harvested using RNeasy Mini Kit (Qiagen, Canada) and analyzed using real-time quantitative PCR (QPCR) as described in (35, 52). Briefly, 1 μg of RNA was reverse-transcribed (RT) to synthesize cDNA using Quantitect Reverse Transcription Kit (Qiagen, Canada). To ensure successful removal of genomic DNA, no-RT reaction was included. 1/20th of RT reaction was used as a template for QPCR amplification using specific primers listed in Appendix G and the FastStart SYBR Green kit (Roche Applied Sciences, Canada) or Promega GoTaq qPCR Master Mix (Promega, WI). Data was acquired using ABI7300 and ABI7500 QPCR (Applied Biosystems, CA) or Eppendorf Realplex2 (Eppendorf, Canada) instruments. Data was normalized to β-actin, analyzed as described in (53), using P19 or P19 control cell line day 0 (monolayer cultures) as a calibrator, and expressed as percent maximum. Data

represents mean ± SEM from at least two independent biological experiments and using two clonal populations per cell line.

### 3.3.6 Northern Blot analysis

RNA from differentiating P19 and P19[Nkx-MEF2C/EnR] cells was harvested using the LiCl/Urea method and analyzed using northern blot procedure as described previously (50). Briefly, a total of 12 μg of RNA for each sample was separated by denaturing gel electrophoresis and transferred to Hybond-N nylon membranes (GE Life Sciences, Canada). The membranes were hybridized with α-32P dCTP labeled MEF2C/EnR and 18S cDNA fragments described previously (21). Results shown are representative of 3 separate clones for each cell line.

### 3.3.7 Immunoblot analysis

Total protein extracts from differentiating mES and P19 EC cells and whole eyes of E16 $Gli2^{+/-}$ heterozygous and $Gli2^{-/-}$ homozygous mice were harvested using RIPA buffer. 10-20 μg of total protein was resolved using 4-12% gradient NUPAGE gels (Invitrogen, Canada) according to manufacturer's protocol using MOPS SDS running buffer. Resolved proteins were transferred to PVDF or nitrocellulose membranes, blocked in 5% milk, and reacted with Gli2 (54), calmodulin binding protein (CBP) (Millipore, MA), MEF2C (Santa Cruz, sc-13266), α-tubulin (Sigma, DM1A) or β-actin specific (Sigma, AC-74) antibodies. Signal was detected using HRP-conjugated secondary anti-rabbit (Millipore, MA), anti-mouse (Cell Signalling, MA) or anti-goat (Santa Cruz, sc-2020) antibodies.

### 3.3.8 Chromatin immunoprecipitation (ChIP) assays

50 μg of chromatin from day 5 differentiating P19[MEF2C] cells and 150 μg of chromatin from day 4 differentiating P19[Gli2] cells was immunoprecipitated using 2 μg of MEF2C specific (Santa Cruz, sc-13266), Gli2 specific (Santa Cruz, G-20) or goat IgG non-specific antibodies (Invitrogen, Canada) as described in (51). Briefly, cells were cross-linked with 4 percent formaldehyde (Fischer Scientific, Canada) and chromatin was sheared as described in (51). Sheared chromatin was incubated with Gli2, MEF2C or IgG antibodies and the immune complexes were captured using protein G sepharose beads as described in (51). Gli2, MEF2C or IgG-bound chromatin was quantified as a percent chromatin input using QPCR analysis as described above. To be considered a true association, each ChIP sample was examined for the enrichment of a chromatin locus immunoprecipitated with a specific antibody and compared to the same chromatin locus immunoprecipitated with a non-specific IgG (ANOVA with $p<0.05$), and compared to a negative control locus, such as the Ascl1 (55) or Hdac4 locus (ANOVA followed by post-hoc Tukey HSD test with $p<0.05$). Data represents mean ± SEM from three independent biological experiments. Primers are listed in Table 3.1.

**Table 3.1 Oligonucleotide sequences of primers utilized for ChIP-QPCR experiments.**

Chr = chromosome, BS = binding site.

| Target gene | Position of BS in mm9 genome | Strand Orien-tation | Forward primer | Reverse Primer |
|---|---|---|---|---|
| *Mef2c* A | Chr13: 83556197 - 83556209 | + | TGAAAAAGGAAATATCCCACTTAGA | TTGCATGGGTTCACACCTAA |
| *Mef2c* B | Chr13: 83589537 - 83589549 | + | AGTTGCCTGAGCCTGTTTTC | TTTTTCGGCAATGATTTTCC |
| *Mef2c* C | Chr13: 83657044 - 83657056 | + | TCTCCAGTTCCTGGGAAGAA | CTTTCGGCTGGAGAGTCTTG |
| *Mef2c* D | Chr13: 83663947 - 83663935 | + | ACACACGCACACTTCGTCTC | GACCCACACAGAACCTTCAAA |
| *Mef2c* E | Chr13: 83734849 - 83734861 | + | TTCCCATTTGGACCATTACC | ACCCACGCACTGAGACTTTC |
| *Mef2c* F | Chr13: 83772819 - 83772831 | + | AACCCCAATCTTCTGCCACT | AAGCTTTCGCTAGACGTGGA |
| *Mef2c* G | Chr13: 83800500 - 83800512 | + | GAGCCCCCTCTCTAATGTCC | TGTGGGCAAGTGTCTTTCTG |
| *Mef2c* H | Chr13: 83803968 - 83803980 | + | CGACCGACCTGCTTTACTTG | AAGTGACATTTGGGGGTCCT |
| *Mef2c* I | Chr13: 83879226 - 83879238 | + | CCTCCCCTCTTGTCAAAGTGT | CCTAATTATTTCAGTTTGGGATGC |
| *Ptch1* A | Chr13: 63667821 - 63667833 | - | TATTGCATGCGAGAGGGTTG | GGAGGGCAGAAATTACTCAGC |
| *Gli1* A | Chr10: 126778677 - 126778689 | - | GCACCCCCTCTCTAGCTTCTATC | GGACCACCCGCGAGAAGCGCAAACT |
| *Ascl1* A | Chr10: 86936603 - 86936615 | - | CCTAAGATCAATGGGCCAAA | CCCACCCAACTGTCCTAGAG |
| *Gli2* A | Chr1: 120906302 - 120906324 | - | AACAGGGTCTCTTCACATAGCC | ATCTGCGGAGCACCTACTGT |
| *Gli2* B | Chr1: 120887621 - 120887643 | - | GCATTTCTAAAGCTTGGTGGA | CCGTGTTAAACATGACTAAAATGAT |
| *Gli2* C | Chr1: 120827230 - 120827246 | - | TGAGTTATTGTTGGCGACTTCA | AAACTGGTGTGCTGGCTAGG |
| *Gli2* D | Chr1: 120812203 - 120812225 | - | CCCATTAACCACTGCTTTGC | GCTATATTTTCCTATTCATGGCATAC |
| *Gli2* E | Chr1: 120799022 - 120799034 | - | CTTCCCTGGGCCATTAAAGT | TGCTAATACTCCCAGGCACA |
| *Gli2* F | Chr1: 120796554 - 120796566 | - | TGCCCTAATTACACAAACAGCA | CCTACTAATAACCTCCATGGATCA |
| *Gata-4* A | Chr14: 63864687 - 63864697 | + | AAGCGCTCTTTTCTCCTTCC | GTGAGGGCTACAGGGAGTGA |
| *MyoG* A | Chr1: 136186098 - 136186104 | + | ATTTGCCCTTCTGGGTTTCT | GCTCAGCAGCACCTTAAACC |
| *Hdac4* A | Chr1: 93901621 - 93901643 | + | ATCTCCCACTGTTGGTCTGC | GGTTTTCACTTTGTGGATTTGG |

### 3.3.9 Co-immunoprecipitation assays

HEK-293 cells (ATCC, #CRL-1573) were seeded at 1 million cells per 100 mm tissue-culture grade dish and 24 h later cotransfected with a total amount of 3 μg of Gli2, MEF2C-Flag or Flag-vector using Fugene (Roche, Canada). 24 h post-transfection, cells were washed twice with ice-cold PBS and lysed with buffer containing 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% TRITON X-100. Lysates were clarified by centrifugation for 15 min at 13,000 g. 0.5 mg of total protein was subjected to FLAG immunoprecipitation according to manufacturer's protocol (Sigma-Aldrich, MO). Bound proteins were eluted by boiling the beads in 1x SDS-PAGE loading buffer for 10 min at 95°C. Transfection efficiency was monitored by co-transfecting GFP and analyzing its fluorescence. All protein extraction and incubation procedures were carried out at 4°C in the presence of 1x protease inhibitor cocktail (Roche, Canada) and 0.5 mM phenylmethanesulfonylfluoride (PMSF).

Eluted proteins from co-immunoprecipitation assays in HEK-293 cells were resolved using 4-12% gradient NUPAGE gels, transferred to PVDF membranes and reacted with Gli2- or MEF2C-specific antibodies as described above. Gli2 purification efficiency was quantified by band densitometry analysis using ImageJ program (56).

### 3.3.10 Reporter assays

Gli2, MEF2C-Flag and GATA-4 expressing plasmids are described elsewhere (25, 47, 57). Gli-responsive promoter is described in (58) and MEF2-responsive promoter is from Affymetrix, CA. The *Nkx2-5* promoter, comprising -3059 to +223 nt relative to the Nkx2-5 transcriptional start site, termed Nkx2-5-luc, is described in (59).

P19 EC cells were plated at a density of 150,000 cells per 35 mm tissue culture grade dish and transiently cotransfected, as described above, 24 h later with a total amount of 3 μg of DNA with or without Gli2, MEF2C-Flag and the Nkx2-5 promoter, Gli-responsive promoter or MEF2-responsive promoter driving luciferase gene in ratios 2:1 relative to luciferase reporters. Transfection efficiency was monitored by transfecting Renilla as described in (51). 24 h after transfection, cells were washed twice with ice-cold PBS and lysed according to Dual Luciferase Kit protocol (Promega, WI). Luciferase activity was assayed using 10-15 μl of lysate and LmaxII384 luminometer (Molecular Devices, USA). To remove background, the normalized activity of the luciferase reporter plasmid alone was subtracted from its activity in the presence of Gli2 and/or MEF2C and GATA-4. The activity of the luciferase reporter in the presence of protein expression plasmids was normalized to the activity in the presence of Gli2 (arbitrarily set at 1). Synergy was calculated as described in (60) according to the following formula:

$$synergy = \frac{RLUs(MEF2C + Gli2)}{RLUs(MEF2C) + RLUs(Gli2)},$$

where relative luciferase units (RLUs) represent luciferase activity normalized to Renilla activity. P19 cells transfected with GATA-4 and Nkx2-5-luc in ratio 2:1 served as a positive control.

### 3.3.11 Bioinformatics analysis

Conserved Gli DNA binding sites and MEF2 DNA binding sites in the Mef2c and Gli2 genes (±100 kb) were identified using Mulan (Multiple Sequence Local Alignment and Visualization tool) as described previously (61). The primers for identified binding sites were designed using Primer 3 software (62) (for primer sequences, see Table 3.1).

Putative genomic targets for both Gli and MEF2 transcription factors were identified using SynoR (Genome miner for synonymous regulation) as described in (63). The distance between neighbouring DNA binding sites was set to a) 4 to 100 bp; b) 100 to 200 bp; c) 200 to 300 bp. The mouse genome (mm9) was used as a base and was compared to the human genome (hg18). Identified genes are listed in Table S1. Functional annotation analysis of the identified potential targets was performed using DAVID (Database for Annotation, Visualization, and Integrated Discovery) software as described in (64, 65).

### 3.3.12 Statistical analysis

ANOVA followed by post-hoc Tukey HSD test was performed using XLSTAT11 software (Addinsoft, NY) to determine statistical significance between mean values of two groups (*, $p<.05$; **, $p<.01$).

## 3.4 Results

### 3.4.1 Gli2 and MEF2C are expressed during cardiomyogenesis in mES and P19 EC cells

Differentiation of mES cells into cardiac muscle is schematically depicted in Fig. 3.1A, using a 7 day aggregation protocol (25), where cardiac muscle forms as early as days 8-9 (Fig. 3.1A). Formation of cardiac muscle from mES cells was confirmed by indirect immunofluorescence with an anti-MHC antibody, MF20 (Fig. 3.1B).

Changes in gene expression during cardiomyogenesis in mES cells were examined for several markers (Fig. 3.1C). Downregulation of Oct-4, a marker of pluripotency in ES cells (66), confirmed that mES cells differentiated efficiently (Fig. 3.1C, panel Oct-4). Notably, a slight upregulation of Oct-4 was observed on day 3, in agreement with a

previously reported role for Oct-4 in cardiac mesoderm formation (67). The mesodermal marker Brachyury T (BraT) (68), was also upregulated on day 3 of mES differentiation (Fig. 3.1C, panel BraT). The highest expression of the cardiac muscle precursor transcription factors Tbx5, Nkx2-5 and GATA-4 (69-71) was detected during days 6-9 of mES differentiation (Fig. 3.1C, panels Tbx5, Nkx2-5 and GATA-4). Finally, the highest expression of the cardiac muscle marker MHC6 was detected on day 9 of mES differentiation (Fig. 3.1C, panel MHC6). During mES differentiation, MEF2C mRNA levels became highly upregulated starting from day 6 (Fig. 3.1D, MEF2C panel), whereas Gli2 mRNA levels were downregulated starting from day 3 (Fig. 3.1D, Gli2 panel). There was a trend in Gli1 mRNA downregulation on days 3-6 of differentiation (Fig. 3.1D, panel Gli1). In contrast, Ptch1 mRNA was upregulated on days 6-9 of mES differentiation (Fig. 3.1D, panel Ptch1). MEF2C and Gli2 protein expression levels correlated with their mRNA expression levels (Fig. 3.1E). MEF2C protein expression was induced after day 3 of differentiation and Gli2 protein expression was downregulated after day 3, although its expression persisted until day 9 (Fig. 3.1E).

To confirm that the upper band on the Gli2 immunoblot did indeed correspond to Gli2 protein, we analyzed whole eye total protein extracts from $Gli2^{+/-}$ heterozygous and $Gli2^{-/-}$ homozygous mice. The only band absent in the $Gli2^{-/-}$ protein sample was the upper band corresponding to ~180 kDa (Fig. 3.1F). Thus, the upper band represents Gli2, which correlates well with previously published data (54, 55) and with the theoretical molecular weight of 165 kDa for full-length mouse Gli2 protein (accession number Q0VGV1). Therefore, both Gli2 and MEF2C proteins were expressed during mES cell cardiomyogenesis (summarized in Table 3.2).

**Figure 3.1. Gli2 and MEF2C are expressed during mES differentiation.**

**(A)**: Schematic representation of cardiomyogenesis in D3 mouse ES cells, as described in Materials and Methods. **(B-E)**: mES cells were differentiated and examined **(B)**: for MHC expression using MF20 antibodies on day 9 of differentiation. Nuclei were stained with Hoechst, scale bar is 30 μM; **(C & D)**: by QPCR analysis for the indicated genes on days 0, 3, 6 and 9 of differentiation, n=3. Error bars represent +/- SEM. (C) Values were compared to day 0 values and statistical significance was determined using ANOVA (*$p<0.05$ and **$p<0.01$). (D) To determine statistical significance between all days analyzed, ANOVA followed by post-hoc Tukey HSD analysis was performed, *$p<0.05$ and **$p<0.01$, n.s.= not significant.; **(E):** Immunoblot analysis of total protein extracts using MEF2C- and Gli2-specific antibodies at the time shown. Arrows designate Gli2 or MEF2C protein band(s), and asterisk denotes non-specific binding of the Gli2 antibodies. α-tubulin served as a loading control. Lanes were spliced out from the same autoradiogram as designated by vertical lines. Gli2 band densities were measured from one representative experiment using ImageJ program (56), normalized to α-tubulin and expressed as percent maximum; **(F):** Immunoblot analysis of eye total protein extracts from E16 $Gli2^{+/-}$ and $Gli2^{-/-}$ mice using Gli2-specific antibodies. Asterisk denotes non-specific binding of the antibodies. β-actin served as a loading control.

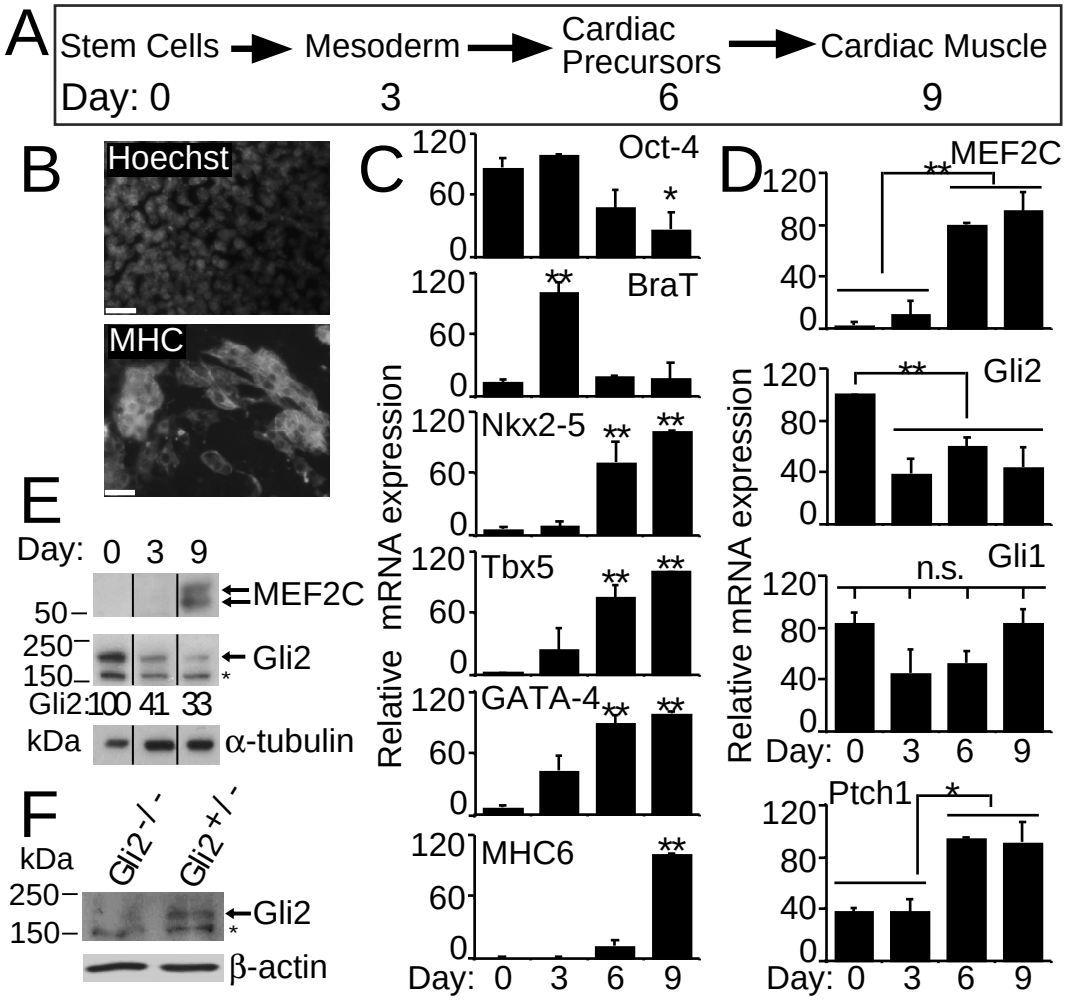

A
Stem Cells
Mesoderm
Cardiac Precursors
Cardiac Muscle
Day: 0
3
6
9
B
Hoechst
MHC
C
Oct-4
BraT
Nkx2-5
Tbx5
GATA-4
MHC6
Relative mRNA expression
Day: 0 3 6 9
D
MEF2C
Gli2
Gli1
Ptch1
n.s.
E
Day: 0 3 9
MEF2C
Gli2
Gli2: 100 41 33
α-tubulin
kDa
F
Gli2 -/-
Gli2 +/-
Gli2
β-actin

**Table 3.2. Summary of gene expression changes in cell lines treated with or without DMSO.**

Cell lines indicated on the left were aggregated under the conditions described in Materials and Methods and the induction of muscle marker gene expression was monitored. (+++ = high expression; ++ = elevated expression; + = normal expression; +/- = downregulated expression, - = not expressed or basal level of expression; N.D. = not determined). The timing of temporal gene expression in parental mES and P19 EC cells is shown. For stable cell lines, the change in expression relative to the respective control cell lines and the day examined, were indicated (D=day).

| Cell line | Treat-ment | BraT | Nkx2-5 | GATA-4 | Tbx5 | MHC6 | Gli2 | MEF2C | Refe-rence |
|---|---|---|---|---|---|---|---|---|---|
| mES | - | + D3 | + D6-9 | + D6-9 | + D6-9 | + D9 | + highest on D0 | + D6-9 | Fig. 3.1 & (74, 75) |
| P19 | DMSO | + D1-3 | + D4-6 | + D3-6 | + D4-6 | + D5-6 | + D4-6 | + D4-6 | Fig. 3.2 & (27) |
| P19[Gli2] | DMSO | +/- D2 | ++ D6 | ++ D6 | ++ D6 | ++ D6 | +++ D0-6 | ++ D4-9 | Fig. 3.3 |
| P19[MEF2C-TAP] | DMSO | + D2 | ++ D6 | ++ D6 | + D6 | + D6 | ++ D4-5 | +++ D0-6 | Fig. 3.3 |
| P19[Gli/EnR] | DMSO | + D0-9 | +/- D4-6 | +/- D4-6 | +/- D4-6 | +/- D4-6 | + D0-9 | +/- D4-6 | Fig. 3.4 & (47, 51) |
| P19[Nkx-MEF2C/EnR] | DMSO | + D2-6 | +/-* D0-6 | - D0-6 | - D6 | - D6 | +/- D6 | +/-* D0-6 | Fig. 3.5 & (21) |
| P19[Nkx/EnR] | DMSO | ++ D2-4 | N.D. | - D0-4, D6 | N.D. | N.D. | N.D. | - D6 | (40) |
| P19 [β-cat/EnR] | DMSO | +/- D0-9 | N.D. | N.D. | N.D. | N.D. | - D0-9 | N.D. | (76) |
| P19[Pax/EnR] | DMSO | ++ D0-9 | N.D. | + D9 | N.D. | N.D. | + D0-9 | N.D. | (77) |
| P19 [Meox/EnR] | DMSO | + D0-9 | + D6 | + D6 | N.D. | N.D. | +/- D0-9 | N.D. | (47) and unpublished observations |
| P19 [MyoD/EnR] | DMSO | N.D. | N.D. | - D9 | N.D. | N.D. | +/- D9 | - D9 | (78) |
| P19[EnR] | DMSO | + D0-6 | + D0-6 | + D0-6 | N.D. | N.D. | + D0-6 | + D0-6 | (40)(153) (153) |

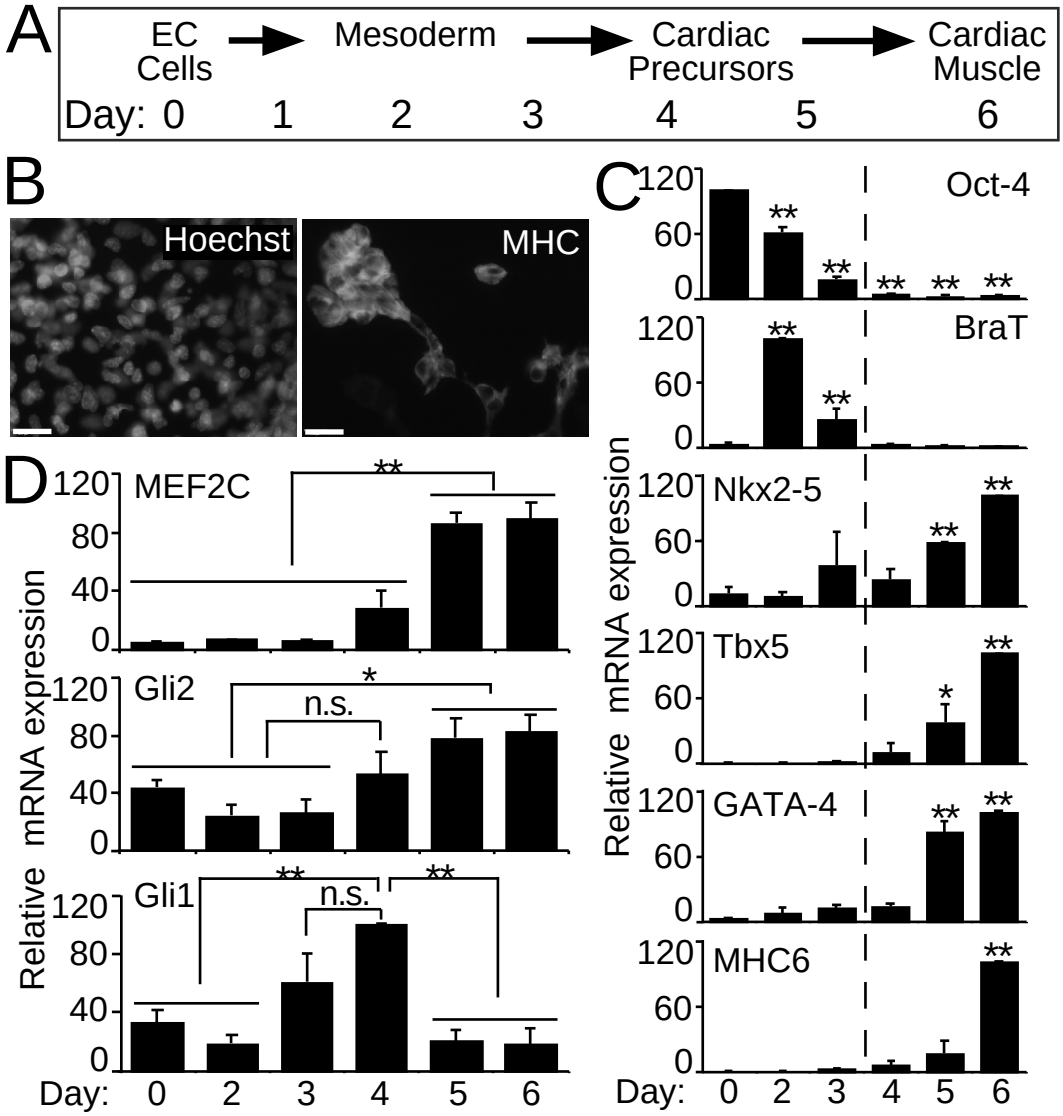
* The expression of Nkx2-5 and MEF2C was elevated during days 0-3, whereas it was downregulated during days 4-6 of P19[Nkx-MEF2C/EnR] differentiation as compared to P19 control cells ((21) and Fig. 3.7C).

Since mES cells spontaneously differentiate into cell types of all three germ layers upon removal of leukemia inhibitory factor (72) and only a small proportion of cells differentiate into cardiac muscle using a standard embryoid body differentiation protocol, it is difficult to specifically correlate the expression profile of MEF2C and Gli2 with cardiac myogenic differentiation in a heterogeneous population of mES cells. P19 EC cells, in contrast, mainly differentiate into mesoderm and endothelial lineages upon addition of DMSO (27, 30-34). Moreover, P19 EC myogenic differentiation is very similar to mES myogenic differentiation (25, 35). For these reasons, we compared the expression pattern of Gli2 and MEF2C during mES differentiation with DMSO-induced differentiation of P19 EC cells.

Cardiomyogenic differentiation of P19 EC cells is schematically presented in Fig. 3.2A. P19 EC cells treated with DMSO successfully differentiated into cardiac muscle on day 6 as observed by indirect immunofluorescence using MHC-specific antibodies (Fig. 3.2B). Gene expression changes during cardiomyogenesis in P19 EC cells were followed by QPCR analysis of myogenic markers (Fig. 3.2C). Downregulation of Oct-4 starting from day 2 indicated the loss of pluripotency in EC cells (Fig. 3.2C, panel Oct-4). Induction of mesoderm is indicated by the robust upregulation of BraT expression on day 2 of differentiation (Fig. 3.2C, panel BraT). Induction of cardiomyogenesis was confirmed by elevated expression of Nkx2-5, Tbx5 and GATA-4 during days 5-6 of P19 EC differentiation (Fig. 3.2C, panels Nkx2-5, Tbx5 and GATA4).

**Figure 3.2. Gli2 and MEF2C are expressed during P19 EC DMSO-induced myogenesis.**

P19 EC cells were differentiated in the presence of 1% DMSO as described in Materials and Methods. **(A):** Schematic representation of cardiomyogenesis in P19 EC cells. **(B):** P19 cells were examined for MHC expression on day 6 of differentiation using MHC-specific antibodies. Nuclei were stained with Hoechst dye. Scale bar is 30 μM; **(C & D):** QPCR analysis of the expression of the indicated genes at the times shown, n=3. Error bars represent +/- SEM; (C): Day 2-6 values were compared to day 0 values and statistical significance was determined using ANOVA (*$p<0.05$ and **$p<0.01$); (D) To determine statistical significance between all days, ANOVA followed by post-hoc Tukey HSD analysis was performed, *$p<0.05$ and **$p<0.01$, n.s.= not significant.


A
EC Cells
Mesoderm
Cardiac Precursors
Cardiac Muscle
Day: 0 1 2 3 4 5 6
B
Hoechst
MHC
C
Oct-4
BraT
Nkx2-5
Tbx5
GATA-4
MHC6
Relative mRNA expression
Day: 0 2 3 4 5 6
D
MEF2C
Gli2
Gli1
n.s.
Relative mRNA expression
Day: 0 2 3 4 5 6

The robust expression of MHC6 on day 6 of differentiation confirmed the formation of cardiomyocytes in EC cells (Fig. 3.2C, panel MHC6). The expression of MEF2C mRNA was significantly elevated ($p<0.01$) during days 5-6 of DMSO-induced differentiation of P19 EC cells (Fig. 3.2D, panel MEF2C), and resembled the temporal pattern observed in mES cells (Fig. 3.1D, panel MEF2C). There was a significant ($p<0.05$) increase in Gli2 mRNA expression during days 5-6 of P19 EC cardiomyogenesis (Fig. 3.2D, panel Gli2). Gli1 mRNA was upregulated on day 4 of differentiation (Fig. 3.2D, panel Gli1). This data correlates with previous reports showing upregulation of MEF2C and Gli2 mRNA expression during P19 EC DMSO-induced myogenesis using Northern Blot analysis (35, 41, 42, 47, 51, 73). Therefore, MEF2C and Gli2 transcription factors are expressed during mES and P19 EC cell cardiomyogenesis (summarized in Table 3.2).

### 3.4.2 Gli2 regulates expression of MEF2C during cardiomyogenesis in P19 EC cells

To study the ability of Gli2 to regulate the expression of MEF2C during cardiomyogenesis in stem cells, we examined P19 cells that stably overexpressed Gli2 (Fig. 3.3). Stable overexpression of Gli2 was confirmed by immunoblot analysis using Gli2-specific antibodies (Fig. 3.3A). Antigenic analysis using MHC-specific antibodies revealed an enhancement in the formation of cardiomyocytes in day 6 differentiated P19[Gli2] cultures (Fig. 3.3B and C). This was confirmed by QPCR analysis, which showed increased levels of Nkx2-5, Tbx5, GATA-4 and MHC6 mRNA in P19[Gli2] cells as compared to P19[Control] cells (Fig. 3.3D, panels Nkx2-5, Tbx5, GATA-4 and MHC6) (summarized in Table 3.2). Enhancement of cardiomyogenesis by Gli2 is consistent with previous findings (3, 41). Notably, overexpression of Gli2 resulted in a slight downregulation ($15 \pm 4\%$) of BraT expression on day 2 of differentiation (Fig. 3.3D, panel BraT). Furthermore, P19[Gli2]

cells overexpressing Gli2 protein showed a statistically significant ($p<0.05$) upregulation of MEF2C mRNA levels on day 4 of DMSO-induced differentiation (Fig. 3.3D, panel MEF2C), similar to results shown in the absence of DMSO (41). There was also an 11-20-fold upregulation of MEF2C protein levels in P19[Gli2] cells on days 6-9 of DMSO-induced differentiation as compared to P19[Control] day 4 (Fig. 3.3A). The increases in MEF2C protein expression on days 6-9 in P19 and P19[Gli2] cells (Fig. 3.3A) correlate with the pattern of MEF2C mRNA expression in P19 cells (compare to Fig. 3.2D), which peaks by day 5. The increase in MEF2C mRNA on day 4 in P19[Gli2] (Fig. 3.3D) cells did not correlate with an obvious increase in MEF2C protein on that day (Fig. 3.3A), possibly due to post-transcriptional regulation of the MEF2C protein (reviewed in (15)). Overall, overexpression of Gli2 upregulates MEF2C mRNA and protein expression while enhancing cardiomyogenesis (summarized in Table 3.2).

Since Gli transcription factors have overlapping functions both *in vivo* and *in vitro* (79-82), the use of a dominant negative fusion protein of Gli2 with the Engrailed repression domain (Gli/EnR) would result in repression of all Gli2-bound regulatory elements and the inability of Gli1 or Gli3 to rescue the phenotype. This approach has successfully been used previously both *in vitro* and *in vivo* (21, 76, 77, 83, 84). Furthermore, overexpression of Engrailed does not interfere with cardiomyogenesis in P19 EC cells (40). Stable overexpression of Gli/EnR (Fig. 3.4C, panel Gli/EnR) resulted in the formation of fewer MHC-positive cardiomyocytes when compared to P19 control cells (Fig. 3.4A and B).

**Figure 3.3. Gli2 upregulates MEF2C expression while enhancing cardiomyogenesis in P19 EC cells.**

P19[Gli2] and P19[Control] cell lines were differentiated in the presence of 1% DMSO. **(A):** Immunoblot analyses of Gli2 and MEF2C protein expression in differentiating P19[Control] and P19[Gli2] cells on days 4, 6 and 9 using Gli2-specific or MEF2C-specific antibodies. Arrow designates Gli2 protein band, and asterisk denotes non-specific binding of the Gli2 antibodies. β-actin served as a loading control. Lanes were spliced out from the same autoradiogram as designated by vertical lines. MEF2C band densities from one representative experiment were measured using ImageJ program (56), normalized to β-actin and expressed as percent maximum; **(B):** Day 6 differentiated P19[Control] and P19[Gli2] cultures were reacted with MHC-specific antibodies. Nuclei were stained with Hoechst dye. Scale bar is 30 μm; **(C):** MHC-positive cardiomyocytes from (B) were counted in 10 random fields and expressed as % of the total number of nuclei, n=4. In total, 13,000 cells were counted; **(D):** QPCR analysis of the expression of the genes indicated at the times shown in P19[Control] (black bars) and P19[Gli2] (grey bars) cultures. Error bars represent +/- SEM from two biological replicas of two clonal populations (n=4). Statistical significance using an ANOVA test was determined between groups of P19[Control] and P19[Gli2] samples analyzed on the same day. For MEF2C expression, ANOVA followed by post-hoc Tukey HSD test was performed to determine statistically significant difference between P19[Control] and P19[Gli2] samples on all days analyzed, *$p<0.05$ and **$p<0.01$.

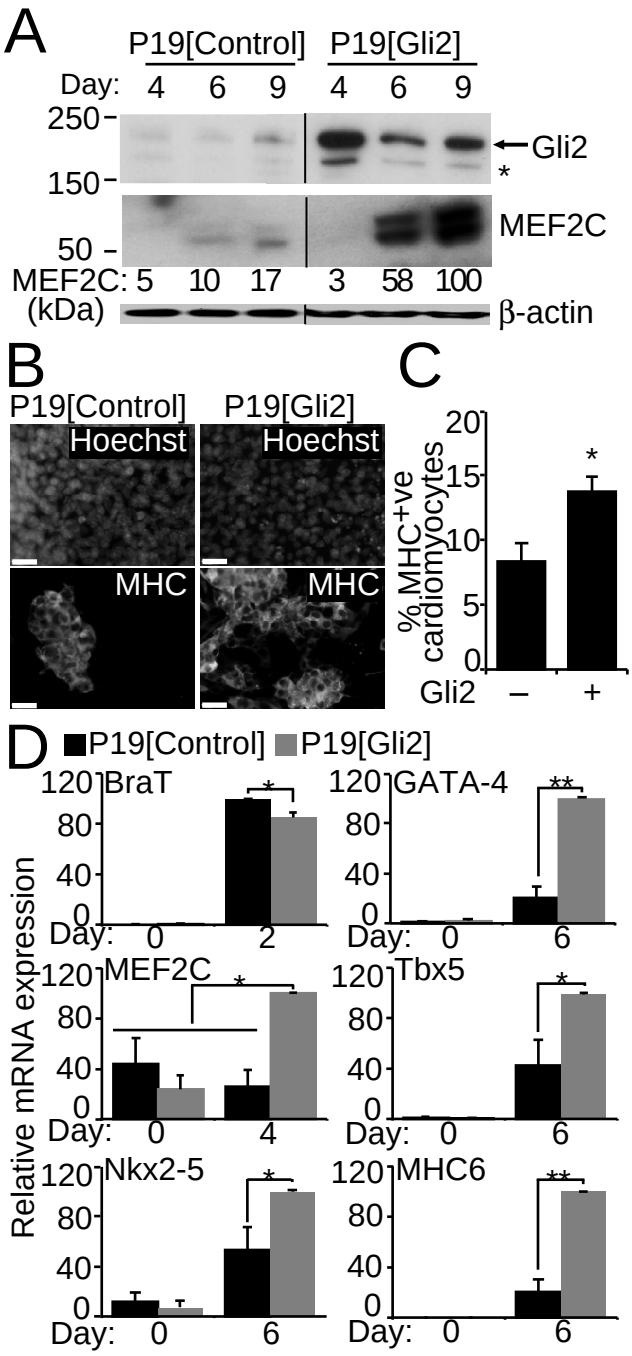
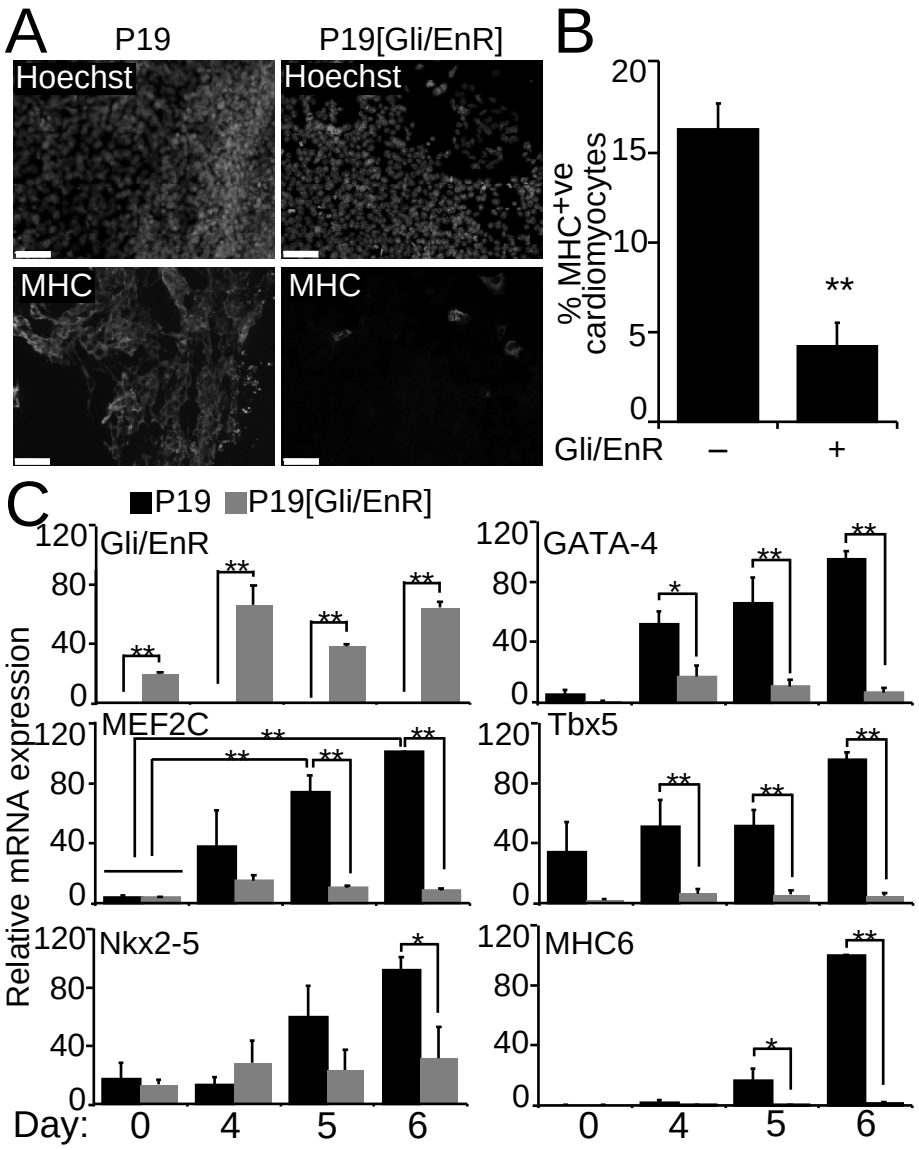

A
P19[Control]
P19[Gli2]
Day: 4 6 9 4 6 9
250
150
50
Gli2
*
MEF2C
MEF2C: 5 10 17 3 58 100
(kDa)
β-actin
B
P19[Control]
P19[Gli2]
Hoechst
Hoechst
MHC
MHC
C
%.MHC+ve cardiomyocytes
20
15
10
5
0
*
Gli2 – +
D
P19[Control]
P19[Gli2]
Relative mRNA expression
BraT
GATA-4
MEF2C
Tbx5
Nkx2-5
MHC6
120
80
40
0
Day: 0 2
Day: 0 6
Day: 0 4
Day: 0 6
Day: 0 6
Day: 0 6
*
**

**Figure 3.4. Expression of Gli/EnR downregulates MEF2C expression while downregulating cardiomyogenesis in P19 EC cells.**

P19 and P19[Gli/EnR] cell lines were differentiated in the presence of 1% DMSO. **(A):** Antigenic analysis of P19 and P19[Gli/EnR] using MHC-specific antibodies on day 9 of differentiation. Nuclei were stained with Hoechst dye. Scale bar is 60 μm; **(B):** MHC-positive cardiomyocytes from (A) were counted in 10 random fields and expressed as % of the total number of nuclei, n=4. In total, 16,000 cells were counted; **(C):** QPCR analysis of the expression of the genes indicated on days 0 and 4-6 in P19 (black bars) and P19[Gli/EnR] (grey bars) cultures. Error bars represent +/- SEM from two biological replicas of two clonal populations (n=4). Statistical significance using ANOVA test was determined between groups of P19 and P19[Gli/EnR] samples analyzed on the same day. For MEF2C expression, ANOVA followed by post-hoc Tukey HSD test was performed to determine statistically significant difference between P19 and P19[Gli/EnR] samples on all days analyzed, *$p<0.05$ and **$p<0.01$.


A
P19
P19[Gli/EnR]
Hoechst
Hoechst
MHC
MHC
B
% MHC+ve cardiomyocytes
20
15
10
5
0
**
Gli/EnR
–
+
C
P19
P19[Gli/EnR]
Relative mRNA expression
Gli/EnR
GATA-4
MEF2C
Tbx5
Nkx2-5
MHC6
120
80
40
0
Day: 0 4 5 6

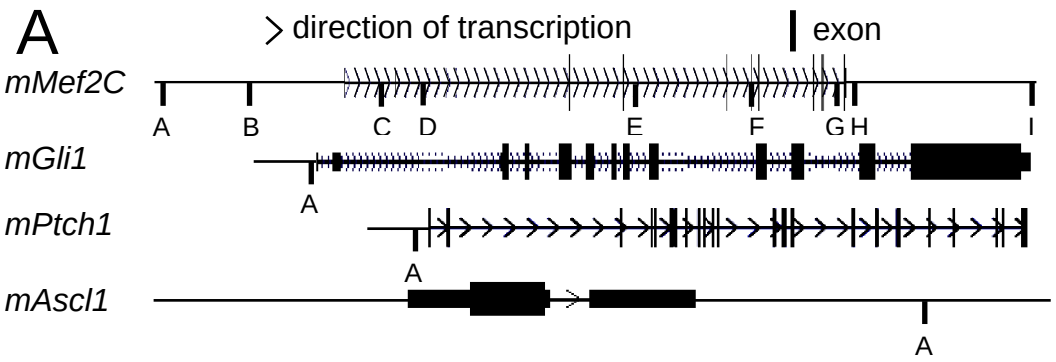
Downregulation of cardiac muscle cell differentiation was confirmed by lower expression of MHC6 (Fig. 3.4C, panel MHC6). The mRNA levels of Tbx5 and GATA-4 were downregulated since day 4, whereas levels of Nkx2-5 were downregulated most significantly ($p<0.05$) on day 6 of P19[Gli/EnR] cellular differentiation (Fig. 3.4C, panels Tbx5, Nkx2-5 and GATA-4). MEF2C mRNA was downregulated in P19[Gli/EnR] cells on days 5 and 6 of differentiation (Fig. 3.4C, panel MEF2C). The effect of MEF2C downregulation is not due to global gene repression by Gli/EnR fusion protein since Gli/EnR did not greatly affect expression of mesodermal markers such as BraT in DMSO-induced differentiation (47) (Table 3.2). P19[Gli/EnR] cells did not lose pluripotency or the ability to differentiate as they were shown to differentiate into glial and neuronal cells in the presence of retinoic acid (55, Chapter 2). Therefore, Gli2, or genes bound by Gli2, is essential for maintaining normal expression of MEF2C during cardiomyogenesis in P19 EC cells (summarized in Table 3.2).

### 3.4.3 Gli2 associates with *Mef2c* gene elements during cardiomyogenesis in P19 EC cells

In order to determine if Gli2 binds *Mef2c* gene elements during cardiomyogenesis, we performed ChIP experiments. In silico analysis of the *Mef2c* gene (±100 kb) revealed 9 conserved theoretical Gli binding sites (GBS) (*Mef2c* A-I, Fig. 3.5A and B, summarized in Table 3.1), suggesting that MEF2C could be a target of a Gli transcription factor. Since the highest expression of Gli2 protein and of MEF2C mRNA was detected on day 4 of P19[Gli2] cellular differentiation (Fig. 3.3A and 3D, respectively), we performed ChIP analysis of day 4 differentiating P19[Gli2] EC cells.

**Figure 3.5. Gli2 associates with *Mef2c* gene elements during cardiomyogenesis *in vitro*.**

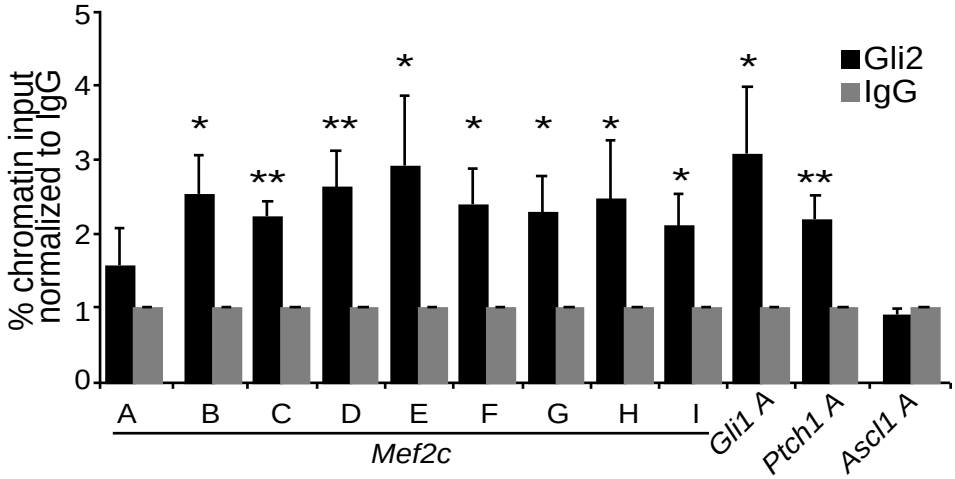
**(A):** Custom tracks of murine *Mef2c*, *Gli1*, *Ptch1* and *Ascl1* genes using UCSC genome browser (http://genome.ucsc.edu). Letters designate conserved Gli binding sites (GBS), their genomic positions are listed in Table 3.1. Listed genes (± 100 kb) from mouse and human genomes were searched for conserved theoretical GBS as described in (61); **(B):** Comparison of mouse and human sequences of *Mef2c* A-I sites from (A). The sequence of the Gli2 binding site (GBS) is marked in bold. **(C):** ChIP analysis of Gli2-bound *Mef2c*, *Gli1*, *Ptch1* and *Ascl1* genes on day 4 of P19[Gli2] cellular differentiation in the presence of 1% DMSO. Black bars designate genomic regions immunoprecipitated with Gli2-specific antibodies, and grey bars designate genomic regions precipitated with IgG-nonspecific antibodies. *Gli1* and *Ptch1* promoters served as positive controls, *Ascl1* gene element served as a negative control. Percent chromatin input was calculated using QPCR analysis and primers listed in Table 3.1. Error bars represent +/- SEM from three biological replicas (n=3). Statistical significance was determined as described in Materials and Methods, *$p<0.05$, **$p<0.01$.




B

| *MEF2C* site | Species | Sequence | % GBS conservation |
|---|---|---|---|
| A | *H. sapiens* | tat**GGACCACCCTGC**act | 100 |
| | *M. musculus* | tat**GGACCACCCTGC**agt | |
| B | *H. sapiens* | gca**TATGGGTGTTCT**gaa | 100 |
| | *M. musculus* | gca**TATGGGTGTTCT**gaa | |
| C | *H. sapiens* | cac**AGTCCACCCACA**gcc | 75 |
| | *M. musculus* | caa**AGCACACCCACT**gcc | |
| D | *H. sapiens* | acg**CGTACACCCAGA**cat | 100 |
| | *M. musculus* | acg**CGTACACCCAGA**cat | |
| E | *H. sapiens* | gtg**CGTGGGTGTTGA**aaa | 100 |
| | *M. musculus* | gtg**CGTGGGTGTTGA**aaa | |
| F | *H. sapiens* | cat**CGACCTCCAAGT**gca | 91.7 |
| | *M. musculus* | cat**AGACCTCCAAGT**gca | |
| G | *H. sapiens* | gcc**AGTAGGTGGTGC**ttt | 100 |
| | *M. musculus* | gcc**AGTAGGTGGTGC**ttt | |
| H | *H. sapiens* | tca**GGACCCCCAAAT**gtc | 100 |
| | *M. musculus* | tca**GGACCCCCAAAT**gtc | |
| I | *H. sapiens* | acg**CCACAACCCACG**tgt | 100 |
| | *M. musculus* | acg**CCACAACCCACG**tgt | |

C



After immunoprecipitation with a Gli2 antibody, a statistically significant enrichment was observed of chromatin fragments, corresponding to *Mef2c* sites B-I (Fig. 3.5C). *Mef2c* site A appeared not to be associated with Gli2 protein (Fig. 3.5C), therefore showing specificity of the ChIP assay. The *Gli1* and *Ptch1* promoter regions were used as positive controls and *Ascl1* gene element was used as a negative control (Fig. 3.5C) based on previous observations (55, 85, 86). From this result, we observe for the first time that Gli2 is associated with *Mef2c* gene elements during cardiomyogenesis in P19 EC cells.

### 3.4.4 MEF2C regulates expression of Gli2 during cardiomyogenesis in P19 EC cells

To study the ability of MEF2C to regulate the expression of Gli2 during cardiomyogenesis in vitro, we examined P19 cells that stably overexpressed MEF2C-TAP (tandem affinity purification tag (25)) (Fig. 3.6). Stable overexpression of MEF2C-TAP was confirmed by immunoblot analysis using CBP-specific antibodies, which recognized the TAP-tag of the MEF2C-TAP protein (Fig. 3.6A). Antigenic analysis using MHC-specific antibodies revealed an enhancement in cardiac muscle formation in P19[MEF2C-TAP] cells when compared to their respective control cells (Fig. 3.6B and C). This is in agreement with previous findings, where MEF2C was shown to induce cardiomyogenesis in the absence of DMSO (45). We also observed significantly ($p<0.01$) increased levels of Nkx2-5 and GATA-4 as well as a trend of increased MHC6 transcripts in day 6 differentiated P19[MEF2C-TAP] cultures as compared to their control cell line (Fig. 3.6D, panels Nkx2-5, GATA-4 and MHC6). Notably, Tbx5 mRNA was not affected by the expression of MEF2C-TAP (Fig. 3.6D, panel Tbx5). Also, expression of MEF2C-TAP did not affect BraT transcript levels, suggesting that MEF2C-TAP affects cardiomyogenesis after mesoderm induction (Fig. 3.6D, panel BraT). Furthermore, overexpression of MEF2C-TAP resulted in

the elevation of Gli2 mRNA expression on day 4 of P19 EC differentiation (Fig. 3.3D, panel Gli2). We also observed an increase in Gli2 protein levels up to 2.4 fold on days 5 and 6 of P19[MEF2C-TAP] differentiation as compared to day 5 of differentiation in P19[TAP] cells (Fig. 3.6A). Therefore, MEF2C upregulates Gli2 expression while enhancing cardiomyogenesis in P19 EC cells (summarized in Table 3.2).

To test if the expression of Gli2 can be regulated by a dominant-negative MEF2C, we examined P19 EC cells expressing MEF2C/EnR under the regulation of the *Nkx2-5* enhancer (Fig. 3.7A). As reported previously (21), P19[Nkx-MEF2C/EnR] cells failed to undergo cardiomyogenesis as evidenced by the absence of MHC- positive cardiac muscle cells (Fig. 3.7B) and a severe downregulation of Nkx2-5, Tbx5, GATA-4 and MHC6 mRNA (Fig. 3.7C, panels Nkx2-5, GATA-4, Tbx5 and MHC6). The phenotype of P19[Nkx-MEF2C/EnR] cells is in accordance with previous reports showing complete absence of cardiomyogenesis *in vivo* or in P19 EC cells (21). Expression of Nkx-MEF2C/EnR affected cardiomyogenesis only, as differentiating P19[Nkx-MEF2C/EnR] cells showed normal upregulation of Pax3 and Myf5 transcripts (data not shown), therefore indicating normal skeletal myogenesis. Gli2 mRNA was downregulated in P19[Nkx-MEF2C/EnR] cells on day 6 of differentiation (Fig. 3.7C, panel Gli2). The loss of Gli2 appears to be specific for a subset of dominant-negative transcription factors, including Nkx-MEF2C/EnR (Fig. 3.7), which caused inhibition of cardiomyogenesis, and MyoD/EnR (78), Meox/EnR (47), and ß-catenin/EnR (76), which caused inhibition of skeletal myogenesis (Table 3.2). In contrast, Gli2 levels were not downregulated in P19 cells expressing Pax/EnR (77), which resulted in the loss of skeletal myogenesis (Table 3.2).

**Figure 3.6. MEF2C upregulates Gli2 expression while enhancing cardiomyogenesis in P19 EC cells.**

P19[MEF2C-TAP] and P19[TAP] cell lines were differentiated in the presence of 1% DMSO. **(A):** Immunoblot analyses of MEF2C-TAP and Gli2 protein expression in differentiating P19[MEF2C-TAP] and P19[TAP] cells on days 5, 6 and 9 using Gli2-specific or CBP-specific antibodies. Arrow designates Gli2 protein band, and asterisk denotes non-specific binding of the Gli2 antibodies. α-tubulin served as a loading control. Lanes were spliced out from the same autoradiogram as designated by vertical lines. Gli2 band densities were measured from one representative experiment using ImageJ program (56) and normalized to α-tubulin. Gli2 band density in P19[TAP] day 5 sample was set at 100%; **(B):** Day 6 differentiated P19[MEF2C-TAP] and P19[TAP] cultures were reacted with MHC-specific antibodies to detect MHC expression. Nuclei were stained with Hoechst dye. Scale bar is 30 μm; **(C):** MHC-positive cardiomyocytes from (B) were counted in 10 random fields and expressed as % of the total number of nuclei, n=4. In total, 14,000 cells were counted; (D): QPCR analysis of the expression of the genes indicated at the times shown in P19[TAP] (black bars) and P19[MEF2C-TAP] (grey bars) cultures. Error bars represent +/- SEM from two biological replicas of two clonal populations (n=4). Statistical significance using ANOVA test was determined between groups of P19[TAP] and P19[MEF2C-TAP] samples analyzed on the same day. For Gli2 expression, ANOVA followed by post-hoc Tukey HSD test was performed to determine statistically significant differences between P19[TAP] and P19[MEF2C-TAP] samples on all days analyzed, *$p<0.05$ and **$p<0.01$.

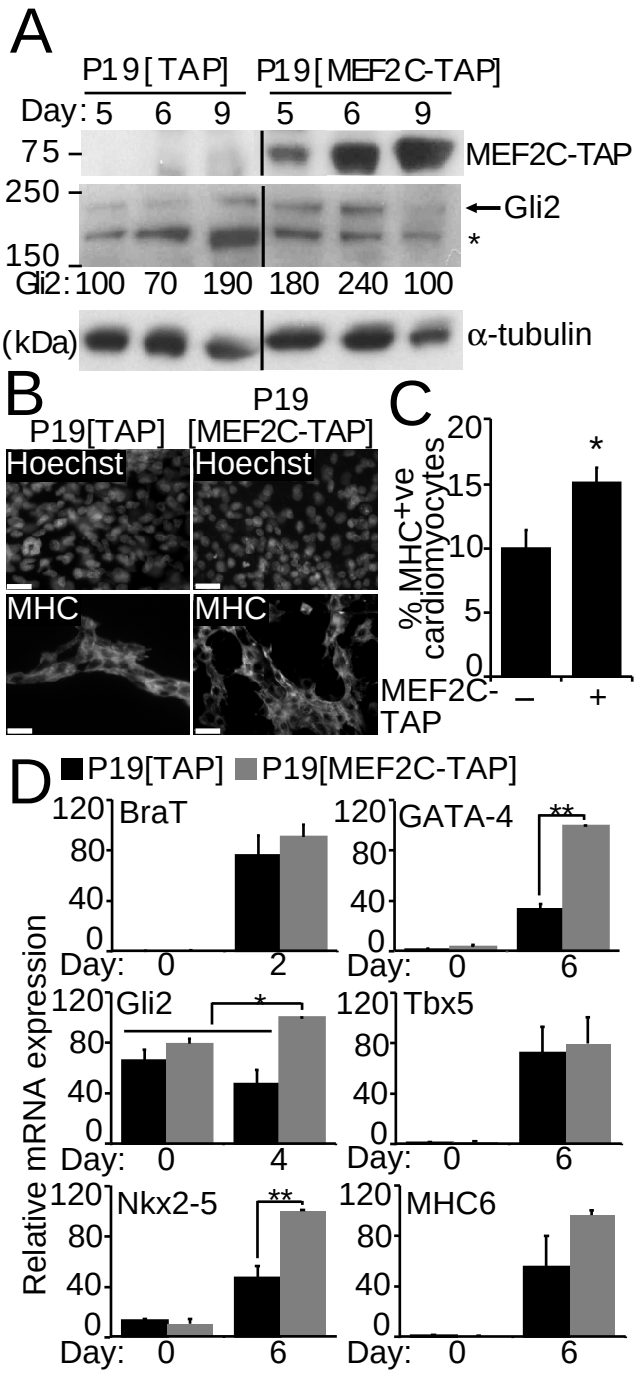
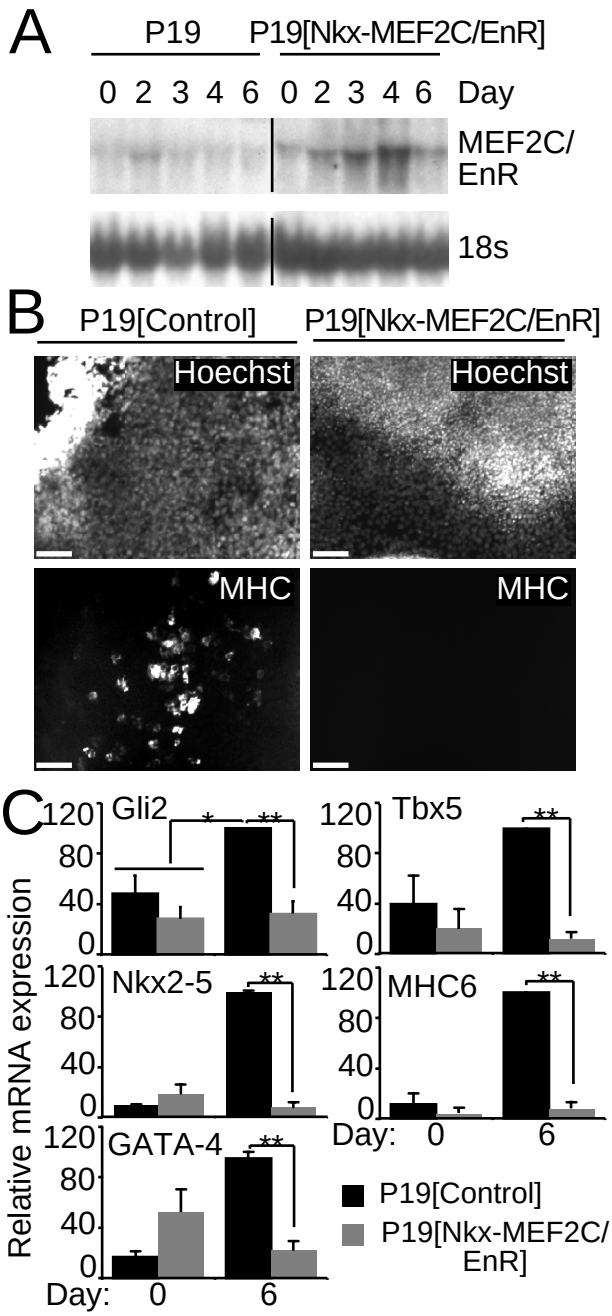

A
P19[TAP]
P19[MEF2C-TAP]
Day: 5 6 9 5 6 9
75
MEF2C-TAP
250
150
Gli2
*
Gli2: 100 70 190 180 240 100
(kDa)
α-tubulin
B
P19[TAP]
P19 [MEF2C-TAP]
Hoechst
Hoechst
MHC
MHC
C
%.MHC+ve cardiomyocytes
20
15
10
5
0
*
MEF2C-TAP
–
+
D
P19[TAP]
P19[MEF2C-TAP]
Relative mRNA expression
BraT
GATA-4
Gli2
Tbx5
Nkx2-5
MHC6
120
80
40
0
Day: 0 2
Day: 0 6
Day: 0 4
Day: 0 6
Day: 0 6
Day: 0 6
*
**
**

**Figure 3.7. Expression of Nkx-MEF2C/EnR downregulates Gli2 expression while inhibiting cardiomyogenesis in P19 EC cells.**

P19[Control] and P19[Nkx-MEF2C/EnR] cell lines were differentiated in the presence of 1% DMSO. **(A):** Northern Blot analysis of the expression of MEF2C/EnR mRNA in P19 and P19[Nkx-MEF2C/EnR] cells on days 0 and 2-6. 18s served as a loading control. Lanes were spliced out from the same autoradiogram as designated by vertical lines. **(B):** Antigenic analysis of P19 and P19[Nkx-MEF2C/EnR] using MHC-specific antibodies on day 6 of DMSO-induced differentiation. Nuclei were stained with Hoechst dye. Scale bar is 60 μm. **(C):** QPCR analysis of the expression of Nkx2-5, GATA-4, Tbx5, MHC6 and Gli2 mRNA in differentiated P19[Control] and P19[Nkx-MEF2C/EnR] cells on days 0 and 6. Error bars represent +/- SEM from two biological replicas of two clonal populations (n=4). Statistical significance using ANOVA test was determined between groups of P19 and P19[Nkx-MEF2C/EnR] samples analyzed on the same day. For Gli2 expression, ANOVA followed by post-hoc Tukey HSD test was performed to determine statistically significant differences between P19 and P19[Nkx-MEF2C/EnR] samples on all days analyzed, *$p<0.05$, **$p<0.01$.


A
P19
P19[Nkx-MEF2C/EnR]
0 2 3 4 6 0 2 3 4 6 Day
MEF2C/
EnR
18s
B
P19[Control]
P19[Nkx-MEF2C/EnR]
Hoechst
Hoechst
MHC
MHC
C
Gli2
Tbx5
Nkx2-5
MHC6
GATA-4
120
80
40
0
*
**
Relative mRNA expression
Day: 0 6
P19[Control]
P19[Nkx-MEF2C/
EnR]

Thus, MEF2C, or genes bound by MEF2C, is essential for maintaining normal Gli2 expression during cardiomyogenesis in P19 EC cells (summarized in Table 3.2).

### 3.4.5 MEF2C associates with *Gli2* gene elements during cardiomyogenesis in stem cells

To test if MEF2C can bind *Gli2* gene elements, *in silico* analysis of the *Gli2* gene (± 100 kb) revealed 6 conserved theoretical MEF2 binding sites (MBS) (*Gli2* A-F, Fig. 3.8A and B, summarized in Table 3.1) suggesting that Gli2 could be a target of MEF2. ChIP analysis of day 5 differentiating P19[MEF2C] cells revealed statistically significant enrichment, with a MEF2C antibody, of chromatin fragments corresponding to *Gli2* A-D and F sites, but not to *Gli2* E site (Fig. 3.8C). The *Hdac4* intron region, which is located on the same chromosome as the *Gli2* gene (summarized in Table 3.1), was not enriched in the ChIP assay with MEF2C antibodies (Fig. 3.8C), therefore serving as a negative control and demonstrating the specificity of ChIP analysis using MEF2C-specific antibodies. The *Gata-4* and *MyoG* promoter regions were used as positive controls (Fig. 3.8C). Therefore, MEF2C is associated with Gli2 gene elements during cardiomyogenesis in P19 EC cells.

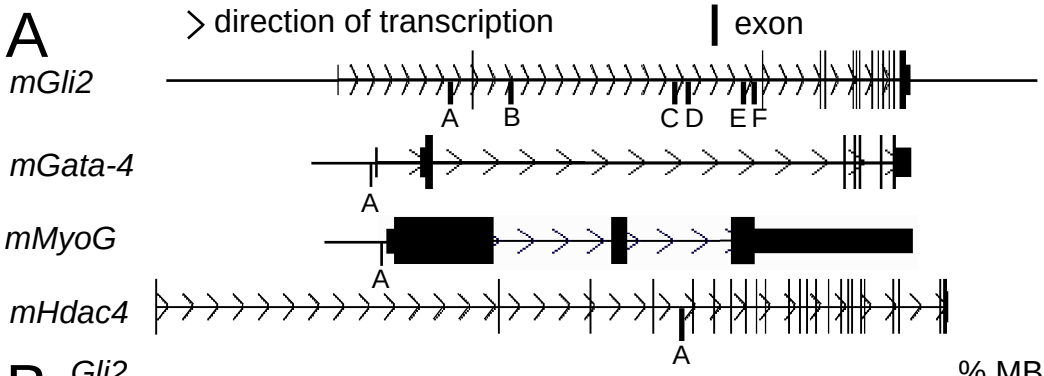
### 3.4.6 Gli2 and MEF2C factors form a protein complex that can function synergistically.

Based on the finding that Gli2 and MEF2C are co-expressed during later stages of cardiomyogenesis in mES and during P19 EC cardiomyogenesis (Fig. 3.1 and 3.2, respectively) and they regulate each other's expression as well as have similar abilities to regulate cardiomyogenesis in P19 EC cells (Fig. 3.3 and 3.6), we hypothesized that these transcription factors may form a protein complex to synergistically regulate the expression of cardiomyogenesis-related genes.

**Figure 3.8. MEF2C associates with *Gli2* gene elements during cardiomyogenesis *in vitro*.**

P19[TAP] and P19[MEF2C-TAP] cell lines were differentiated in the presence of 1% DMSO. **(A):** Custom tracks of murine *Gli2*, *Gata-4*, *MyoG* and *Hdac4* genes using UCSC genome browser (http://genome.ucsc.edu). Letters designate conserved MEF2 binding sites (MBS), their genomic positions are listed in Table 3.1. Listed genes (± 100 kb) from mouse and human genomes were searched for conserved theoretical MBS as described in (61); **(B)** Comparison of mouse and human sequences of *Gli2* A-F sites from (A). The sequence of the MEF2 binding site (MBS) is marked in bold. **(C):** ChIP analysis of MEF2C-bound *Gli2*, *Gata-4*, *MyoG* and *Hdac4* genes during P19[MEF2C] cellular differentiation. Black bars designate genomic regions immunoprecipitated with MEF2C-specific antibodies, and grey bars designate genomic regions precipitated with IgG-nonspecific antibodies. *Gata-4* and *MyoG* promoters served as positive controls, *Hdac4* gene element served as a negative control. Percent chromatin input was calculated using QPCR analysis and primers listed in Table 3.1. Error bars represent +/- SEM from three biological replicas (n=3). Statistical significance was determined as described in Materials and Methods, *$p<0.05$, **$p<0.01$.

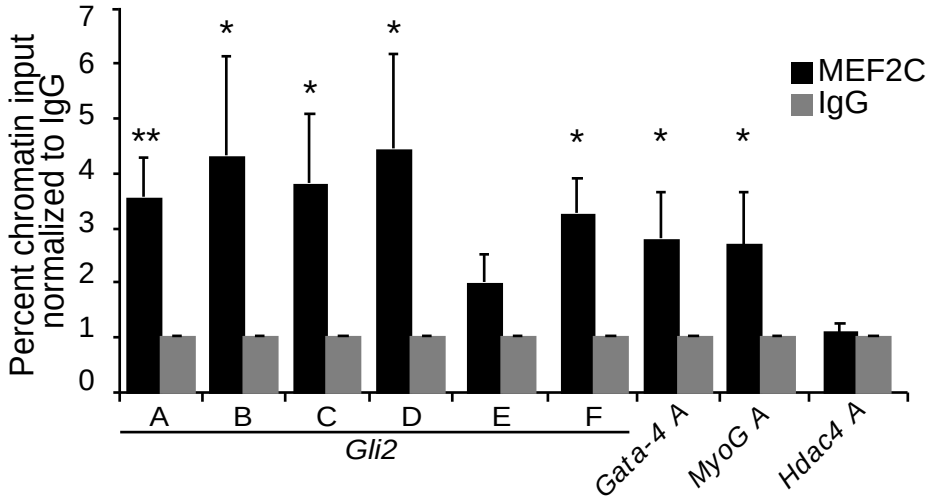
A




B

| Gli2 site | Species | Sequence | % MBS conservation |
|---|---|---|---|
| A | *H. sapiens* | ttt**TTGTATTTTTATTAGAGACAGG**gtt | 77.3 |
| | *M. musculus* | ttt**TGGTTTTTTTTTTAAAAACAGG**gtc | |
| B | *H. sapiens* | tgt**CAAAACTAATTTTAGCATTTCT**aaa | 86.3 |
| | *M. musculus* | tgt**CAGGACTAATTTTAGCATTTCT**aaa | |
| C | *H. sapiens* | aaa**TGAGTTATTGTTGGAG**act | 93.8 |
| | *M. musculus* | aaa**TGAGTTATTGTTGGCG**act | |
| D | *H. sapiens* | tgt**GCCTAATTTATAAATTGAACTT**tat | 90.9 |
| | *M. musculus* | tgt**GCCTGATTTATAAATTGAACCT**tat | |
| E | *H. sapiens* | ctc**ATAAAAATATCC**tcc | 91.67 |
| | *M. musculus* | ccc**ATAAAAATACCC**tct | |
| F | *H. sapiens* | ggg**CCCATTTTTAA**cgc | 100 |
| | *M. musculus* | gag**CCCATTTTTAA**tgc | |

C

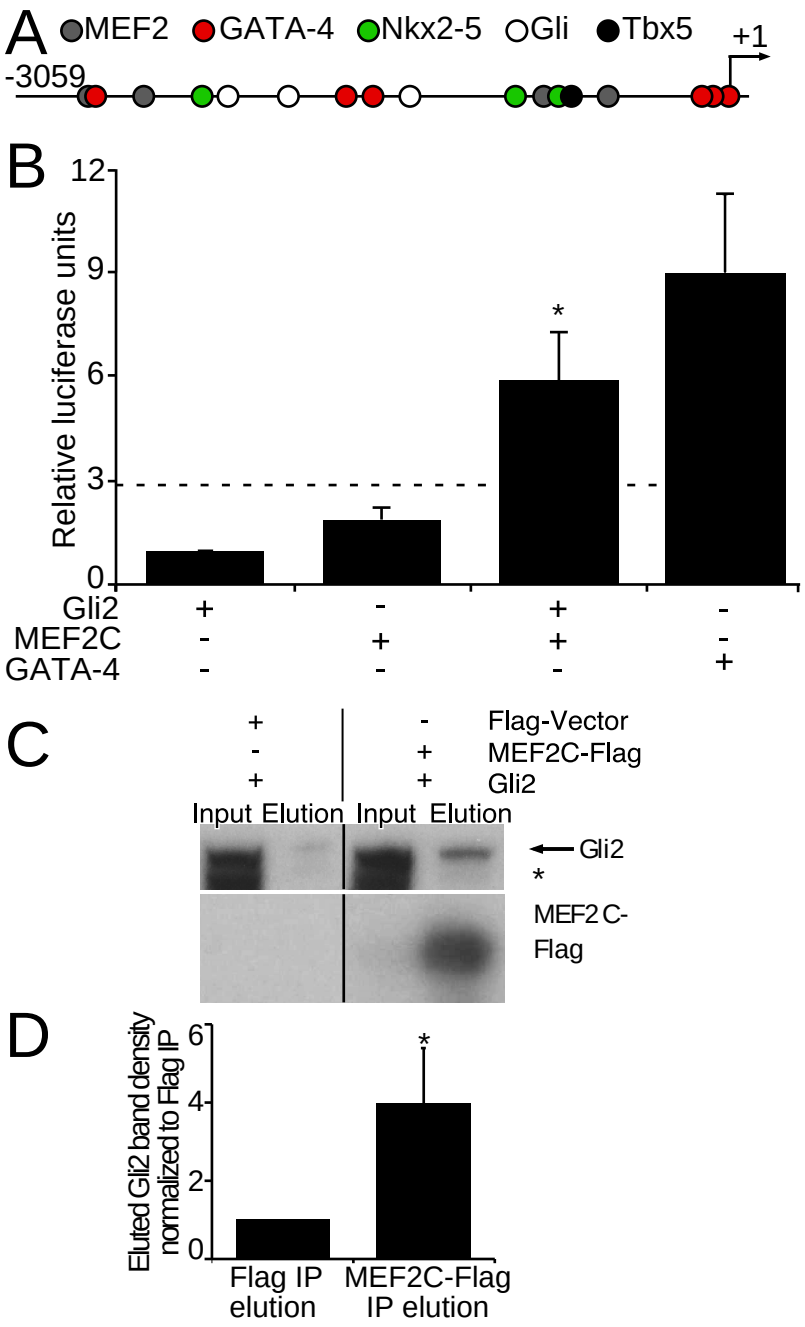


To test this hypothesis, we first explored the ability of Gli2 and MEF2C to synergize in luciferase reporter assays. To this end, we took advantage of an endogenous *Nkx2-5* promoter (-3059 to +223 nt relative to Nkx2-5 transcriptional start site), which was shown to drive gene expression in the cardiac crescent at E7.25, as well as outflow tract and right ventricle at E10.5, and require combinatorial function of multiple regulatory factors (59). This *Nkx2-5* promoter contained 3XGli, 3XMEF2, 6XGATA-4, 3XNkx and 1XTbx5 binding sites and was fused to the luciferase gene (Nkx2-5-luc) (59) (Fig. 3.9A). When Gli2 and MEF2C were co-transfected together, they synergistically activated the *Nkx2-5* promoter up to 6 ± 2 fold as compared to the theoretical additive value of 3 ± 0.3 fold change (Fig. 3.9B, dashed line). The activation of the *Nkx2-5* promoter by Gli2 and MEF2C together was similar to that of GATA-4 alone (Fig. 3.9B). When Gli2 and MEF2C were co-transfected with luciferase reporters containing either 5xGli or 5xMEF2 binding sites alone, no synergy or additive effect was observed (data not shown). Therefore, Gli2 and MEF2C synergistically activate the *Nkx2-5* promoter.

To test if Gli2 and MEF2C formed a protein complex, we co-transfected Gli2 and MEF2C-Flag or Flag-vector into HEK-293 cells. Gli2 co-immunoprecipitated with MEF2C-Flag, but not with the Flag-tag alone (Fig. 3.9C). Analysis of autofluorescence of co-transfected GFP was performed to ensure that all samples were transfected with equal efficiency. Quantitative analysis of bands corresponding to Gli2 protein revealed a 4 ± 1 fold higher amount of Gli2 co-immunoprecipitated with MEF2C-Flag when compared to co-IP with Flag-vector (Fig. 3.9D). Therefore Gli2 and MEF2C proteins physically associate and synergistically activate transcription.

**Figure 3.9. Gli2 and MEF2C form a protein complex and synergize on the *Nkx2-5* promoter.**

**(A):** Schematic representation of Nkx2-5-luc reporter construct. Circles designate Gli (white), MEF2 (grey), Nkx (green), GATA-4 (red) and Tbx5 (black) binding sites; +1 designates transcriptional start site (TSS); -3059 designates the beginning of the *Nkx2-5* promoter relative to TSS; **(B)** Gli2 and MEF2C were transfected alone or together with or without Nkx2-5-luc in P19 cells. Cells transfected with GATA-4 served as a positive control. Error bars represent +/- SEM from ten biological replicas (n=10), *$p<0.05$. The dashed line represents the value at which Gli2 and MEF2C function additively. Transfection efficiency was monitored by assaying activity of co-transfected Renilla luciferase. Synergy and statistical significance was calculated as described in Materials and Methods. The average activation of Nkx2-5-luc by Gli2 was 2.3 ± 0.4 (data not shown) and was normalized to 1; **(C):** Gli2 was co-immunoprecipitated with MEF2C-Flag but not with Flag-vector when transfected in HEK-293 cells. Transfection efficiency was monitored by assaying autofluorescence of co-transfected GFP. Arrow designates Gli2 protein band, and asterisk denotes non-specific binding of the Gli2 antibodies. Lanes were spliced out from the same autoradiogram as designated by vertical lines. **(D):** Quantification of Gli2 band density from elution fractions in (C) using ImageJ program. Error bars represent +/- SEM from three biological replicas (n=3), *$p<0.05$.


A
MEF2
GATA-4
Nkx2-5
Gli
Tbx5
+1
-3059
B
Relative luciferase units
12
9
6
3
0
*
Gli2
MEF2C
GATA-4
C
Flag-Vector
MEF2C-Flag
Gli2
Input Elution Input Elution
Gli2
*
MEF2C-Flag
D
Eluted Gli2 band density normalized to Flag IP
6
4
2
0
*
Flag IP elution
MEF2C-Flag IP elution

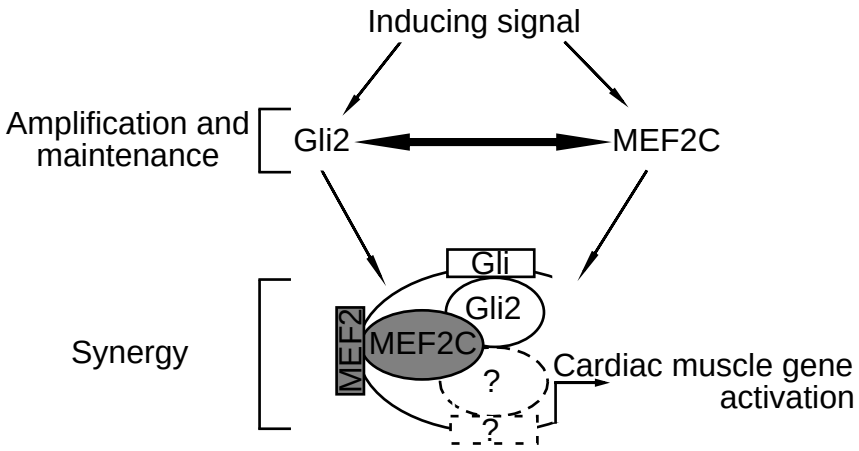
### 3.5 Discussion

In this chapter, we have shown that Gli2 and MEF2C are associated with each other's gene elements and are able to regulate each other's expression during cardiomyogenesis in P19 EC cells. Further, Gli2 and MEF2C form a protein complex capable of synergistically activating gene promoters that participate in cardiomyogenesis. Thus, we propose a model similar to that for MEF2 and MRFs (reviewed in (15)), where Gli2 and MEF2C transcription factors induce and maintain each other's expression, as well as form a protein complex that synergizes on promoters containing both Gli and MEF2 binding elements during cardiomyogenesis (Fig. 3.10). It is likely that other transcription factors participate in the Gli2-MEF2C protein complex, designated as "?".

Our results obtained *in vitro* provide added mechanistic insight into the roles of Gli2 and MEF2C in cardiomyogenesis. Whereas only cardiac outflow tract anomalies were detected in the developing hearts of $Gli2^{-/-}Gli3^{+/-}$ mice (6, 14), $MEF2C^{-/-}$ mice had heart looping defects along with gross abnormalities in the right ventricle as well as in the outflow tract (19, 20). This suggests that Gli2 and MEF2C may function co-operatively during cardiac outflow formation. Our studies are consistent with an overlapping pattern of defects that could be explained by Gli2 and MEF2C having both shared and distinct subsets of targets, likely dependent upon the unique protein complexes formed in each case.

**Figure 3.10. Gli2 and MEF2C interact during cardiomyogenesis *in vitro*.**

We propose a model in which Gli2 and MEF2C induce each other's expression and bind each other's gene elements (designated by thick arrow) as well as form a protein complex on gene promoters capable of synergistically activating their expression. Other protein(s) participating in the Gli2/MEF2C protein complex are designated by "?".


Inducing signal
Amplification and maintenance
Gli2
MEF2C
Gli
Gli2
MEF2
MEF2C
Synergy
?
?
Cardiac muscle gene activation

The results from this chapter suggest that both Gli2 and MEF2C may be active at the cardiac muscle progenitor stage. This is supported by the embryonic expression patterns of MEF2C (starting from E7.5) (16, 17) and Gli1 (E7.0-8.0) (3, 10), which are both expressed after mesodermal markers BraT (E6.5) and MESP1 (E6.5), and concomitantly with the cardiac progenitor marker Nkx2-5 (E7.0) (reviewed in (87)). Although expression of Gli2 resulted in downregulated expression of BraT, the decrease was minimal (15 ± 4%) in comparison with the upregulation of expression of other factors (Fig. 3.3D). Furthermore, overexpression of MEF2C, Nkx-MEF2C/EnR and Gli/EnR did not affect the expression of BraT in P19 EC cellular differentiation ((21, 41, 45, 47) and Fig. 3.6D), while affecting the expression of cardiac muscle progenitor markers Nkx2-5 and GATA-4 ((21, 41, 45) and Fig. 3.4C, 6D and 7C).

The temporal patterns of MEF2C and Gli2 expression we identified in both P19 EC and mES cells are consistent with previous reports. MEF2C mRNA is upregulated during cardiomyogenesis in mES cells by day 6, after expression of mesodermal markers BraT and Mesp1/2 (74) and in P19 EC cells from days 6-7 (27), similar to our findings (Fig. 3.1 and 3.2). Much less information is available about the expression of Gli transcription factors during ES myogenesis. It is known that Shh signalling members are expressed in undifferentiated hES cells (88) as well as in cardiomyocytes derived from P19 EC (41), P19CL6 (43) and mES cells stably expressing neomycin-resistance gene under the regulation of cardiac α-myosin heavy chain promoter (23). A further complication is that Gli2 is expressed in numerous lineages, including brain, bone, cartilage, muscle, lung, and pancreas (reviewed in (89)). In addition, Gli2 might be involved in maintaining stem cell pluripotency in ES cells as it was shown to directly regulate expression of the pluripotency markers Sox2

(90) and Nanog (91). Thus, it is possible that a high level of Gli2 expression is necessary to maintain the pluripotent state of ES cells, and lower level of Gli2 expression is sufficient to support mES differentiation. This phenomenon has been shown previously for Oct-4 and Sox2, which play roles in maintaining pluripotency (66, 92) as well as in directing early stages of ES cell differentiation (67, 93). Finally, the primary effect of the Hedgehog signalling is the activation of Gli2 protein function, as opposed to the upregulation of Gli2 mRNA or protein expression (94, 95). The elevated expression of Ptch1, marker of active Hedgehog (Hh) signalling (96), during days 6-9 of mES differentiation (Fig. 3.1D), suggests that the Hh signalling pathway is active when MEF2C is expressed in differentiating mES cells (Fig. 3.1D).

In comparison to mES cells (72, 97), DMSO-treated P19 EC cells differentiate into only a limited number of mesodermal and endodermal lineages (27, 30-34). In this system, Gli2 expression was upregulated during cardiomyogenesis from days 5-6 (Fig. 3.2D), in agreement with previous studies (41, 42). Furthermore, Gli2 was significantly upregulated in the same temporal pattern as MEF2C (Fig. 3.2D). In summary, P19 EC cells provide a good model system for examining the functional interaction between Gli2 and MEF2C.

It was previously demonstrated that Gli2 induced the expression of MEF2C and cardiomyogenesis in P19 EC cells in the absence of DMSO (41). Recently, it was shown that the activation of the Shh signalling pathway upregulated MEF2C mRNA transcript levels in the second heart field *in vivo* after chick embryos were treated with Shh signalling pathway activator, SAG agonist, at HH14 (98). Drosophila embryos expressing mutant loss-of-function MEF2 protein showed downregulated expression of Ci (Cubitus Interruptus, a single Drosophila homolog of vertebrate Gli transcription factors) (99). Our results support

and extend previous results by showing that Gli2 and MEF2C can upregulate each other's expression while enhancing DMSO-induced cardiomyogenesis in P19 EC cells, suggesting the possibility that they function in a regulatory loop.

Gli2 and MEF2C enhanced each other's mRNA expression on day 4 of differentiation, and not in undifferentiated cells (day 0). We have previously shown that MyoD, a master-regulator of skeletal myogenesis capable of converting fibroblasts into skeletal myocytes (100), induced skeletal myogenesis in P19 EC cells only upon cellular aggregation (101, 102). Indeed, all of the muscle transcription factors studied to date in P19 EC cells required cellular aggregation to be functional (21, 40, 45-47, 50, 52, 73, 76-78, 101-103). It is therefore not surprising that the expression levels of Gli2 and MEF2C were not affected in undifferentiated (day 0) P19 stable cell lines.

The expression of dominant-negative Gli2 resulted in downregulation of MEF2C expression and cardiomyogenesis. To our knowledge, this is the first indication that expression of Gli/EnR results in impaired cardiomyogenesis in P19 EC cells. Previous reports showed that Shh signalling is not essential for cardiomyogenesis during embryogenesis (3, 4) or in P19 EC cells (42), although a reduction or delay of cardiomyogenesis was observed. However, treatment of a cardiac lineage-restricted subline of P19 cells, P19CL6 cells, with the Shh signalling inhibitor cyclopamine blocked their differentiation into beating cardiac myocytes (43). The difference in the latter results may be due to a lack of compensatory signalling molecules, derived from other lineages, implicated in activating Gli factors, such as TGFβ (104), Fgf (105) and Wnt (106). Finally, Zic factors have also been shown to bind genomic Gli binding sites and to modulate the transcriptional activity of Gli transcription factors (107). Our use of a dominant-negative approach would

override compensatory activating signals (47, 55), showing a role for Gli2 function during cardiomyogenesis.

While ubiquitously expressed Gli2 and MEF2C each enhanced cardiomyogenesis (Fig. 3.3 and Fig. 3.6), and ubiquitously expressed Gli/EnR impaired cardiomyogenesis (Fig. 3.4), surprisingly pgk-driven MEF/EnR enhanced cardiomyogenesis (46) and skeletal myogenesis (unpublished observations). This is likely due to the dual nature of MEF2 factors, which have the ability to recruit HDAC4/5 to genes, inhibiting transcription (108), as well as recruiting HATs, activating transcription (108). A repressive role for Gli2 in regulating entry into various lineages was not observed, since the expression of Gli/EnR under the pgk promoter resulted in abolished skeletal myogenesis (47) and attenuated levels of neurogenesis and gliogenesis (55, Chapter 2) at a specific stage of each respective differentiation program (47, 55). Therefore, expression of Gli2/EnR under a general promoter such as pgk, impaired the formation of different stem cell differentiation pathways, including cardiomyogenesis, at specific stages.

While overexpression of MEF2C-TAP upregulated transcript levels of Gli2, Nkx2-5 and GATA-4, it did not affect Tbx5 mRNA expression. This correlates with previous studies, where knockdown of MEF2C using morpholinos disrupted zebrafish heart tube looping, but did not affect Tbx5 expression (109). It is possible that other MEF2 family proteins were able to rescue the expression of Tbx5 in zebrafish (109). Genetic redundancy of MEF2C family members is supported by our finding that the expression of Tbx5, Gli2 and cardiac muscle progenitor markers was drastically reduced in the presence of dominant-negative Nkx-MEF2C/EnR *in vitro*, and the loss of heart formation *in vivo* (21). It is likely that the *Nkx2-5* enhancer driving the expression of MEF2C/EnR can be regulated by Gli2 and the

previously described regulatory loop between MEF2C, GATA-4, and Nkx2-5 (21, 40, 45, 73). Thus, as Nkx2-5 is expressed during formation of cardiac muscle progenitors, the overexpression of Nkx-MEF2C/EnR will inhibit the function of endogenous MEF2C and Gli2, as well as other cardiogenic factors, resulting in the loss of cardiomyogenesis. This is supported by the fact that both endogenous Gli2 and MEF2C were downregulated, but not abolished, by day 6 in P19[Nkx-MEF2C/EnR] cells, while the expression of Nkx-MEF2C/EnR was reduced ((21) and Fig. 3.7).

We report for the first time that Gli2 and MEF2C associate with each other's gene elements. Although existing literature supports identification of Gli2 as a target of MEF2C by demonstrating that the *Drosophila* MEF2 protein directly bound gene elements of Ci (a Drosophila homolog of vertebrate Gli transcription factors) (99), MEF2C was not previously reported to be a target of Gli3 in murine developing limb in a genome-wide ChIP-on-chip analysis (110). It is possible that MEF2C is a target of the Gli transcription factors at an earlier time-point than the time-point analyzed in the study (E11.5) (110). Members of the Shh signalling pathway are expressed starting from E8.0 in murine paraxial mesoderm (81), and MEF2C is the first MEF2 family factor to be expressed in somites starting from E8.5-9.0 (16). Finally, it is possible that MEF2C is a target of Gli2, and not Gli3, during cardiomyogenesis.

Notably, the majority of the Gli2-bound sites are located in the intron regions of the *Mef2c* gene. It was previously shown that there are multiple promoter and enhancer regulatory elements within the *Mef2c* gene introns (17, 111, 112). Of those known regulatory elements in the *Mef2c* gene, the *Mef2c* D site, which was bound by Gli2 in this study, is located approximately +500 bp relative to the 3' end of the reported cardiac promoter (17).

Thus, it is possible that other *Mef2c* gene elements bound by Gli2 are located within novel regulatory regions, however, their identification is outside the scope of this study.

Much like the *Mef2c* gene sites bound by the Gli2 protein, *Gli2* sites bound by the MEF2C protein are located in the intron regions of the Gli2 gene. Although regulatory elements in the *Gli2* gene have not yet been reported, it is possible that its introns contain novel uncharacterized regulatory regions. For example, the *Gli2* B site, which was bound by MEF2C in this study, is part of the predicted genomic enhancer, which is well conserved between human, mouse, chicken, frog and fugu genomes ((113), element hs1790). Although this region was not found to have any enhancer activity at E11.5 when transfected into mouse embryos in a reporter cassette driving β-gal expression ((113), element hs1790), it is possible that it can act as an enhancer at an earlier or later time point in development.

Both Gli2 and MEF2C are capable of enhancing the development of cardiac muscle through upregulation of the Nkx2-5, GATA-4 and BMP-4 regulatory loop (this chapter and (41, 45)), skeletal muscle via upregulation of MRFs (47, 48) and neurons through enhancement of Ascl1/Mash1 expression (55, 114) in pluripotent P19 EC cells. The ability of Gli2 and MEF2C to regulate a similar subset of differentiation pathways in pluripotent P19 EC cells, led us to hypothesize that Gli2 and MEF2C could function together. We showed that Gli2 and MEF2C form a protein complex that functions synergistically on the *Nkx2-5* promoter. MEF2 proteins are known to form synergistic protein complexes with cardiac muscle factors such as Hand1 (115) and GATA-4 (116). More recently, MEF2C, together with GATA-4 and Tbx5, was shown to convert mouse fibroblasts into cardiac myocytes (117). Furthermore, our findings are consistent with Pitx2, which is a homeodomain transcription factor involved in heart development, and MEF2 protein complex studies,

where both MEF2 and Pitx2 DNA binding sites were necessary for the synergistic action of the protein complex (118).

*In silico* analysis of the mouse genome identified 957 genes containing both Gli and MEF2 conserved DNA binding clusters (Supplemental File S1). Functional annotation analysis of these potential targets revealed a number of categories enriched amongst Gli and MEF clusters containing genes, such as cell differentiation, regulation of gene expression, chromatin organization, as well as development of nervous system, heart, the skeletal system and muscle organ (Table 3.3). While *Nkx2-5* was not identified as a target gene for Gli2 and MEF2C, probably due to the presence of non-conserved Gli and MEF2 binding elements and/or due to stringent restrictions used in the screening process, *MEF2C* was identified as one of the potential target genes for both Gli and MEF2 transcription factors (Table 3.3). The identification of numerous potential Gli and MEF2 targets regulating various lineages implies that the Gli2/MEF2C interactions identified here may be applicable to other lineages and require further study.

**Table 3.3. Selected gene ontology biological processes significantly enriched among genes containing Gli and MEF2 conserved DNA binding clusters in UTR, promoter, intron and coding sequence regions.**

Altogether, 957 potential target genes were analyzed as described in Materials and Methods. The background set of genes used was the entire mouse genome. For complete list of genes see Supplemental File S1.

| Category | Targets | Fisher Exact P-value | Example genes |
|---|---|---|---|
| Cellular Process | 562 | 2.1E-17 | E2F4, Glis1, CCNB2, Hdac9, RBBP4, TCF4 |
| System Development | 189 | 6.1E-19 | Tbx15, Fgf10, GSK3b, VEGFC, Sox6, Bmp1, Ar |
| Regulation of gene expression | 172 | 6.4E-7 | DNMT3A, POU4F2, Smarcc2, FOXA2, FOXD3, HOXD10, Lhx2, Pbrm1 |
| Cell differentiation | 143 | 4.5E-13 | ANGPT1, Bmp7, Fgf1, PAX2, Ptch1, Hhip, Twist1 |
| Nervous System Development | 99 | 3.3E-15 | BDNF, NRXN1, FOXP2, Gfra1, HOXB3 |
| Embryonic Development | 68 | 3.5E-8 | Notch2, Pdgfra, Pcgf2, HOXD10, ITGA8, Nr4a3 |
| Neurogenesis | 60 | 8.5E-9 | Ntng1, NAV1, Lhx8, APP, Ctnna2 |
| Skeletal System Development | 31 | 9.1E-5 | Bmp5, Sox5, HOXB8, Tapt1 |
| Chromatin Organization | 31 | 5.2E-4 | MYST4, Phf15, ASH1L, ING2, Rnf2 |
| Heart Development | 27 | 4.9E-5 | MEF2C, FOXP1, TTN, Hdac5, ALPK3 |
| Muscle Organ Development | 17 | 1.5E-2 | Dmd, Utrn, Rora, CACNG2 |
| Vascular Smooth Muscle Contraction | 16 | 5.1E-4 | Cacna1C, Cacna1D, BRAF, Prkca |

In summary, we propose a novel mechanistic model, in which Gli2 and MEF2C transcription factors induce and maintain each other's expression as well as form a protein complex capable of synergizing on gene promoters containing both Gli and MEF2 binding elements during cardiomyogenesis *in vitro* (Fig. 3.10). This is the first evidence that the Sonic Hedgehog signalling pathway is directly linked to the function of MEF2C protein. Our findings are similar to the MEF2 and MRF interaction model (reviewed in (15)). The Gli2/MEF2C protein complex may function during the induction of several lineages, including cardiac and skeletal muscle, as well as neurons. This is supported by the identification of numerous putative targets for both Gli2 and MEF2C, which are enriched in neurogenesis, as well as muscle organ and heart tube developmental processes (Table 3.3). Understanding the complex network of transcription factors that regulates lineage determination during stem cell differentiation is important for potential future cell therapies.

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### 3.7 Authors' contributions

A.V. carried out mES, parental and stable P19 EC differentiation, immunofluorescence, QPCR analysis, ChIP assay with Gli2 antibodies, IP from HEK-293 cells, bioinformatics analysis and manuscript writing. A.M. created and differentiated P19[TAP] and P19[MEF2C-TAP] stable cell lines and carried out ChIP assay with MEF2C antibodies. A.F. participated in ChIP assay with Gli2 antibodies. M.S. participated in differentiation and QPCR analysis of mES cells. C.K. differentiated P19[Nkx-MEF2C/EnR] cells, carried out Northern Blot and immunofluorescence analysis of this cell line. I.S.S. conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript. The authors indicate no potential conflicts of interest.

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# CHAPTER 4

# HEDGEHOG SIGNALLING REGULATES MYOD EXPRESSION AND ACTIVITY

## 4.1 Abstract

The inhibition of MyoD expression is important for obtaining muscle progenitors that can replenish the satellite cell niche during muscle repair. Progenitors could be derived from either embryonic stem cells or satellite cells. Hedgehog (Hh) signalling is important for MyoD expression during embryogenesis and adult muscle regeneration. To date, the mechanistic understanding of MyoD regulation by Hh signalling is unclear. Here, we demonstrate that the Hh effector, Gli2, regulates MyoD expression and associates with *MyoD* gene elements. Gain- and loss-of-function experiments in pluripotent P19 cells show that Gli2 activity is sufficient and required for efficient MyoD expression during skeletal myogenesis. Inhibition of Hh signalling reduces MyoD expression during satellite cell activation *in vitro*. In addition to regulating MyoD expression, Hh signalling regulates MyoD transcriptional activity and MyoD activates Hh signalling in myogenic conversion assays. Finally, Gli2, MyoD and MEF2C form a protein complex, which enhances MyoD activity on skeletal muscle-related promoters. We therefore link Hh signalling to the function and expression of MyoD protein during myogenesis in stem cells.

## 4.2 Introduction

The ability to repair and regenerate muscle using stem cells would be beneficial for patients with muscle damage or wasting. Myogenic stem cells can be derived from either embryonic stem cells (1, 2) or adult muscle satellite cells (SC) (reviewed in (3)). To define novel cell therapies for skeletal muscle, it is important to understand molecular mechanisms regulating stem cell formation, proliferation, and differentiation, which generally recapitulate mechanisms controlling their *in vivo* counterparts. Since Hedgehog (Hh) signalling is important for *in vivo* myogenesis (4-7), we set out to define the mechanisms regulating Hh signalling during myogenesis *in vitro*.

The formation of skeletal muscle during embryogenesis and adult muscle regeneration is regulated by two transcription factor families, myocyte enhancer factor 2 A-D (MEF2A-D) and myogenic regulatory factors (MRFs), MyoD, Myf5, MyoG and MRF4 (reviewed in(3)). The inhibition of MyoD and/or Myf5, expression in muscle satellite cells is crucial for the efficient replenishment of the satellite cell niche, a critical step during muscle repair (8-11). Whereas MEF2 proteins are known to directly regulate the expression and function of MyoD (reviewed in (12, 13)), the molecular mechanisms by which Hh signalling controls MyoD expression and its role in regulating MyoD transcriptional activity are currently unknown.

In the developing somite, Myf5 and MyoD are expressed at E8.5 in the dermomyotome and E10.5 in the myotome, respectively (14, 15). While $MyoD^{-/-}$ or $Myf5^{-/-}$ $MRF4^{-/-}$ mice do not show any muscle development defects, $MyoD^{-/-}Myf5^{-/-}MRF4^{-/-}$ mice are devoid of muscle (16, 17), indicating a functional redundancy between MRF members, likely due to binding to similar “E-box” consensus sequences (18). MyoD can convert non-muscle

cell types like fibroblasts into muscle (19, 20) and is often referred to as a master regulator of skeletal myogenesis (21). Importantly, MRFs induce and co-operate with the MEF2 protein family, forming a positive regulatory loop controlling myogenesis (reviewed in (12, 13)).

In mammals, the Hh signalling proteins Sonic (Shh), Indian (Ihh), and Desert (Dhh) bind to the Patched1 (Ptch1) cell surface receptor, leading to de-repression of Smoothened (Smo) activity and regulation of gene expression via nuclear translocation of the Gli transcription factors. Primarily, Gli1 and Gli2 act as transcriptional activators, whereas Gli3 is a transcriptional repressor (reviewed in (22)). During embryonic skeletal muscle formation, Gli factors are expressed as of E8.0 in murine pre-myogenic mesoderm (6). Shh, expressed by the notochord, is sufficient and essential for MyoD expression in the avian somite (23, 24). Similarly, $Shh^{-/-}$ mice fail to express epaxial MyoD and Myf5 (4) and fail to form epaxial myotome (5, 6). In the hypaxial myotome, Shh signalling maintains Myf5 and MyoD expression in mouse limb buds (7, 25, 26). Whereas the molecular mechanism of MyoD expression by Shh signalling is currently not clear, Gli2 is known to directly regulate Myf5 expression by binding to its early epaxial enhancer (EEE) (27). Therefore, Hh signalling is important during embryonic skeletal myogenesis and it directly regulates the expression of at least one MRF member, Myf5.

During adult muscle regeneration, SCs exit the quiescent state, marked by expression of Pax7 (28, 29), and become activated to express MyoD and Myf5 (30). They proliferate, generate myoblasts, and terminally differentiate by inducing expression of MyoG and muscle structural proteins (31), fusing with damaged fibers (reviewed in (3, 32)). Shh, expressed in SCs, is important for MyoD and Myf5 activation during skeletal muscle regeneration (33), and promotes proliferation and differentiation of cultured primary myoblasts (34).

In tissue culture, the expansion of SCs leads to irreversible upregulation of MRF expression (reviewed in (3)), reducing the efficiency of repopulation of the satellite cell niche (9-11). Moreover, SCs isolated from MyoD$^{-/-}$ mice exhibit better engraftment than wild-type myoblasts (8). Thus, the development of cell culture methods to proliferate SCs without triggering the upregulation of the MRFs would greatly enhance the possibility of cell therapy to repair and regenerate skeletal muscle. Myocardin and MEF2 factors are key regulators of adult muscle regeneration (35) and skeletal MLCK (myosin light chain kinase), which regulates MEF2C activity, is important for MyoD expression during SC activation *in vitro* (36). However, the mechanism by which Hh signalling regulates MyoD expression in activated SCs is currently not known.

Muscle progenitors can also be derived from embryonic stem (ES) cells (1, 2) to repopulate the SC niche (1, 37). ES and embryonal carcinoma (EC) cell differentiation are good models of early mammalian embryogenesis (38-40). P19 EC cells, isolated from teratocarcinomas created by injecting E7.5 mouse embryo cells into mouse testes (41), contribute to tissues in live-born chimeric mice (42) and differentiate primarily into cardiac and skeletal muscle upon differentiation in tissue culture in the presence of dimethyl sulfoxide (DMSO) (39). The results from P19 cells have been confirmed in ES cells (2, 36, 43) and in the embryo (44, 45), and P19-derived skeletal muscle shows similar cell morphologies to embryonic and ES-derived muscle, expressing embryonic-specific isoforms of several genes (2, 39).

During skeletal myogenesis in P19 cells, Gli2 is detected starting in pre-myogenic mesoderm, before the expression of MRFs in committed myoblasts (43, 46), similar to the embryo (6). Gli2 regulates the expression of MRFs in P19 cells as shown by loss- and gain-

of-function studies (46, 47), however the mechanism of this regulation remains to be determined. Thus, P19 cells represent a chemically controlled model of ES differentiation, suitable to study the molecular regulation of early skeletal myogenesis by Gli2.

While it is clear that Shh plays an important role in MyoD regulation both during embryonic myogenesis (4, 7) and adult muscle regeneration (33), the molecular mechanism by which this occurs is not fully understood. In this paper, we demonstrate that Gli2 associates with *MyoD* gene elements, while enhancing skeletal myogenesis in P19 cells and activates the MyoD promoter *in vitro*. Further, inhibition of Hh signalling results in reduced MyoD and MEF2C expression both in C2C12 myoblasts and during SC activation. Finally, we show that Hh signalling regulates MyoD protein function, probably via complex formation with MEF2C and Gli2. We therefore directly link the Hh signalling pathway to the expression and function of MyoD protein during skeletal myogenesis in stem cells.

## 4.3 Materials and Methods

### 4.3.1 Plasmids

Gli2, Gli/EnR, MEF2C-TAP, MEF2C-Flag and MyoD-Flag expressing plasmids were described elsewhere (36, 46, 48). The *MyoG* promoter construct was described in (49). The *Chrna1*, *Atp2a1*, *MyoD* CER, PRR and *-2.2kb MyoD* promoter constructs were generated by PCR amplification of their respective promoter sequences and insertion into pGL3b (Promega, WI).

### 4.3.2 Bioinformatics analysis

Conserved Gli DNA binding sites in the *MyoD* gene (±100 kb) were identified as described previously (50) and their binding by MyoD/MyoG in C2C12 cells was identified

from ENCODE/Calbiotech dataset as described in (51). The primers for identified binding sites were designed using Primer 3 software (52) (for primer sequences, see Table 4.2). Genes containing clusters of Gli, MEF2 and MyoD conserved binding sites between mouse (mm9) and human (hg18) genomes were identified using SynoR (Genome miner for synonymous regulation) software as described in (53). The distance between neighbouring DNA binding sites was set to a) 4 to 500 bp; b) 500 to 1000 bp. Identified genes are listed in Supplemental Excel File S2. Functional annotation analysis of the identified putative targets was performed using DAVID (Database for Annotation, Visualization, and Integrated Discovery) software as described in (54, 55). Selected gene ontology biological processes are listed in Table 4.3.

### 4.3.3 Transgenic mice

PtchlacZ$^{+/-}$ mice (56) were maintained on a C57BL/6 background. All animal use was approved by the University of Ottawa Animal Care Ethics Committee and was in accordance with the guidelines of the Canadian Council on Animal Care.

### 4.3.4 Satellite cell isolation and culture

The hind limb muscles were isolated from 3-week-old C57BL/6 or PtchlacZ$^{+/-}$ mice as described (36). Briefly, the isolated muscles were digested with collagenase for 2h at 37°C. SCs were isolated by passing the digested muscle tissue through 70 μm cell strainer and subsequent incubation for 2h in tissue culture plates to remove fibroblasts. The SCs were seeded on matrigel-coated (Invitrogen, Canada) plates at 5,200 cells/cm$^2$ in growth media with 30% FBS without additional growth factors. Cultures of satellite cells were found to contain 75 ± 8% of Pax7$^+$ cells by immunofluorescence staining. On a daily basis, cells were

treated with SB203580 (Calbiochem, Canada) dissolved in DMSO (5 μM), cyclopamine or KAAD-cyclopamine (TRC, Canada) dissolved in methanol (2.5, 5, 10 or 15 μM), and mouse IgG (Invitrogen, Canada) or 5E1 Shh-specific antibodies (6 μg/ml) based on (57). Shh-specific antibodies were purified from the supernatant of 5E1 Shh hybridoma cell line according to the recommendations by the Developmental Studies Hybridoma Bank, IA.

### 4.3.5 P19 cell culture

P19 cells (ATCC, #CRL-1825) were cultured and differentiated as described in (58). Briefly, cells were aggregated in the presence of DMSO for 4 days and formed aggregates were plated onto 0.1%-gelatin covered coverslips or adherent tissue-culture grade dishes for additional 5 days. P19 cells overexpressing various factors including P19[Gli2], P19[Gli/EnR], P19[MEF2C-TAP], P19[Control] or P19[TAP] cells were described elsewhere (36, 46).

### 4.3.6 C3H10T1/2 myogenic conversion assays

C3H10T1/2 cells (ATCC, # CCL-226) were cultured as recommended by the ATCC. For myogenic conversion assays, the cells were seeded at 5,200 cells/cm$^2$ and allowed to grow in complete growth medium containing 10% FBS for 48h. The cells were then transfected using Turbofect according to the manufacturer's protocol (Fermentas, Canada). The amount of DNA typically included 0.9 μg of pRL-Sv40 (Promega, WI) and different combinations of 2 μg of MyoD-Flag, MEF2C-Flag, MEF2C-TAP, Gli2 or Gli/EnR expressing plasmids. Transfection efficiency was monitored by analyzing Renilla activity using Dual Luciferase Kit as per manufacturer's instructions (Promega, WI). 24h after transfection, the media was changed to 2% horse serum with or without KAAD-cyclopamine

(TRC, Canada) at a final concentration of 10 μM based on (59). Methanol was used as a vehicle-control. After an additional 2 days in culture, cells were harvested for total RNA purification using RNeasy purification kit as described in manufacturer's protocol (Qiagen, Canada).

### 4.3.7 C2C12 cell culture

C2C12 myoblast cells (ATCC, #CRL-1772) were grown for 24h in 10% FBS-containing complete growth media until 25% confluence. The media was then changed and contained 10% FBS and 5 or 10 μM KAAD-cyclopamine (TRC, Canada). Methanol was used as a vehicle-control. After 48h in culture, cells were lysed in RIPA (radioimmunoprecipitation) buffer containing 1x protease inhibitor cocktail (PIC) (Roche, Canada) and 0.5 mM phenylmethanesulfonylfluoride (PMSF) (Sigma Aldrich, MO) for immunoblot analysis.

### 4.3.8 Immunofluorescence

On day 9 of P19 cell differentiation, cultures were fixed and incubated with MF20 monoclonal antibody supernatant to detect expression of MHC as previously described (58). MHC indirect immunofluorescence was captured using Leica DMI6000B (Leica Microsystems GmbH, Germany). Images were collected at 400x magnification using Hamamatsu Orca AG camera (Hamamatsu Photonics, Germany) and processed using Velocity 4.3.2 (Perkin Elmer, Canada) software.

On day 1 of SC culture, cells were fixed with ice-cold acetone as described in (60) and incubated with Pax7 monoclonal antibody supernatant (Developmental Studies Hybridoma Bank, IA) and/or β-gal specific antibodies (Aves Lab Inc, OR). Cy3- or

Alexa647-conjugated secondary antibodies (Jackson Immuno Research Laboratories, USA) were used to detect indirect immunofluorescence. Hoechst dye was used to detect nuclei. Pax7 and β-gal indirect immunofluorescence was captured using Zeiss LSM 510 Meta Confocal Microscope at 200x magnification. Images were processed using Zeiss ZEN software (Zeiss, Germany).

### 4.3.9 β-gal staining

On day 1 and day 3 of SC culture, cells were fixed with 4% formaldehyde (Fisher Scientific, Canada) and 0.5% glutaraldehyde (Sigma Aldrich, Canada) for 4 min on ice. After washing with PBS, cells were incubated with β-gal staining solution (0.1% X-gal, 6 mM $K_4[Fe(CN)_6]$•3H2O, 4 mM $K_3[Fe(CN)_6]$, 2 mM $MgCl_2$, 0.01% Na deoxycholate, 0.02% Igepal in PBS) overnight at 37°C as described in (61). The cells were then washed with PBS and mounted in 50% glycerol/50% PBS. Images were collected using Nikon Eclipse 80i microscope (Nikon Intsruments Inc., Canada) and captured using Micro.Publisher 5.0 RTV camera (QImaging, Canada). Images were processed using Image Pro Plus (Media Cybernetics, MD) and Canvas 9 (ACD Systems International Inc., Canada) software.

### 4.3.10 Quantitative PCR analysis

Total RNA from P19, satellite cells or C3H10T1/2 fibroblasts cells was harvested using RNeasy Kit (Qiagen, Canada) or E.Z.N.A. kit (Omega, Canada) and analyzed using real-time quantitative PCR (QPCR) as described in (60). Briefly, 250-500 ng of RNA was reverse-transcribed (RT) to synthesize cDNA using Quantitect Reverse Transcription Kit (Qiagen, Canada). 1/40th of RT reaction was used as a template for QPCR amplification using specific primers listed in Appendix G and the FastStart SYBR Green kit (Roche

Applied Sciences, Canada) or Promega GoTaq qPCR Master Mix (Promega, WI). Data was acquired using ABI7300 and ABI7500 QPCR (Applied Biosystems, CA) or Eppendorf Realplex2 (Eppendorf, Canada) instruments. Data was normalized to β-actin or cyclophilin B and analyzed as described in (62). For P19 cellular differentiations data represents mean ± SEM from at least two independent biological experiments and using two clonal populations per cell line and is expressed as percent maximum. For SCs treated with cyclopamine, SB and Shh-specific antibodies data represents mean ± SEM from at least three animals and is expressed as percent of day 3 of untreated cells. For C3H10T1/2 cells, data represents mean ± SEM from at least four biological replicates and is expressed as percent relative to cells transfected with MyoD.

### 4.3.11 Immunoblot analysis

Total protein extracts from C2C12 cells were harvested using RIPA buffer. 10-20 μg of total protein was resolved using 4-12% gradient NUPAGE gels (Invitrogen, Canada) according to manufacturer's protocol using MOPS SDS running buffer. Resolved proteins were transferred to PVDF or nitrocellulose membranes, blocked in 5% milk, and reacted with MEF2C- (Santa Cruz, sc-13266), MyoD- (BD Biosciences, NJ) or α-tubulin- (Sigma Aldrich, MO) specific antibodies. Signal was detected using HRP-conjugated secondary anti-rabbit (Millipore, MA), anti-mouse (Cell Signalling, MA) or anti-goat (Santa Cruz, sc-2020) antibodies.

### 4.3.12 Chromatin immunoprecipitation (ChIP) assays

150 μg of chromatin from day 4 differentiating P19[Gli2] cells was immunoprecipitated using 2 μg of Gli2 specific (Santa Cruz, G-20) or goat IgG non-specific

antibodies (Invitrogen, Canada) as described in (63). Briefly, cells were cross-linked with 4 percent formaldehyde. Sheared chromatin was incubated with Gli2 or IgG antibodies and the immune complexes were captured using protein G sepharose beads (Invitrogen, Canada). Gli2 or IgG-bound chromatin was quantified as a percent chromatin input using QPCR analysis as described above. Data represents mean ± SEM from three independent biological experiments. Primers are listed in Table 4.2.

### 4.3.13 Co-immunoprecipitation assays

P19[MEF2C-TAP] and P19[TAP] cells were differentiated as described above until day 8. Cells were washed twice with ice-cold PBS and fractionated to obtain nuclear extract. Cytoplasmic extract was obtained by dounce homogenization of the cells in buffer containing 25 mM HEPES, pH 7.8, 1.5 mM MgCl2, 10 mM KCl, 0.1% Igepal and 1 mM dithiothreitol. Nuclear extract was obtained by collecting nuclei by gentle centrifugation at 1 krpm for 10 min and resuspending in sonication buffer containing 50 mM HEPES, pH 7.9, 140 mM NaCl and 0.1% Igepal. Resuspended nuclei were sonicated using a Sonic Dismembrator (Fisher Scientific, Canada) for a total of 5 x 4 second pulses (1 minute rest between pulses) and lysates were clarified by centrifugation at 13,000 g for 25 min. Total nuclear protein from clarified lysates was quantified using Bio-Rad Protein Assay (Bio-Rad, Canada) and 1.1 mg of nuclear protein was incubated with 50 µl of streptavidin resin slurry (Agilent Technologies, TX) overnight. Beads were washed twice with sonication buffer, and bound proteins were eluted by boiling the beads in 1x sample SDS buffer for 10 min at 95°C.

C3H10T1/2 cells were transfected with MyoD-Flag, MEF2C-TAP and Gli2 expressing plasmids as described above. 24h after transfection, cells were incubated for additional 24h in starvation media. The cells were then washed twice with ice-cold PBS and

lysed with buffer containing 50 mM Tris HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, and 1% TRITON X-100. Lysates were clarified by centrifugation for 15 min at 13,000 g. 0.2 mg of total protein was subjected to FLAG immunoprecipitation according to manufacturer's protocol (Sigma Aldrich, MO). Bound proteins were eluted by boiling the beads in 1x SDS-PAGE loading buffer for 10 min at 95°C. Transfection efficiency was monitored by co-transfecting GFP and analyzing its autofluorescence. All protein extraction and incubation procedures were carried out at 4°C in the presence of protease inhibitor cocktail (Roche, Canada) and 0.5 mM PMSF (Sigma-Aldrich, Canada).

Eluted proteins from co-immunoprecipitation assays in C3H10T1/2 or P19 cells were resolved using 4-12% gradient NUPAGE gels, transferred to PVDF membranes and reacted with Gli2- (64), MEF2C-, MyoD- or Flag- (Sigma Aldrich, MO) specific antibodies as described above. The density of Gli2-specific bands in the immunoblot of P19 TAP-IP was performed using ImageJ software as described in (65).

**4.3.14 Reporter assays**

P19 cells were plated at a density of 8,000 cells/cm$^2$ and transiently cotransfected 24 h later using Fugene (Roche, Canada) with or without Gli2, MyoD-Flag, MEF2C-Flag and *Atp2a1* promoter driving luciferase gene. The amount of DNA typically included 125 ng of reporter DNA and Renilla Luciferase pRL-Sv40 (Promega, WI). Gli2, MyoD-Flag, MEF2C-Flag expressing plasmids were transfected at a ratio 2:1 relative to the *Atp2a1* reporter construct.

C3H10T1/2 cells were plated at density of 5,200 cells/cm$^2$ and transiently co-transfected 48h later as described above with or without Gli2, MyoD-Flag, MEF2C-Flag expressing plasmids and *MyoD* (-2.2kb promoter), *MyoD* CER+PRR, *MyoG* or *Chrna1*

reporter constructs. The amount of DNA typically included 125 ng of reporter DNA and pRL-Sv40. For Gli2-mediated activation of *MyoD* -2.2 kb promoter, cells were harvested and assayed 24h after transfection. To study the effect of cyclopamine on MyoD and/or MEF2C transcriptional activity, transfected cells were incubated in the presence of 10 μM KAAD-cyclopamine or methanol (vehicle-control) in starvation media for an additional 24h.

24 or 48h after transfection, luciferase activity was assayed using Dual Luciferase protocol according to manufacturer's instructions (Promega, WI). The activity of the Firefly luciferase was assayed using 10-15 μl of lysate on LmaxII384 luminometer (Molecular Devices, USA) and normalized to activity of co-transfected Renilla. In C3H10T1/2 cells, the normalized activity of luciferase reporter plasmid in the presence of transcription factors was expressed as fold change over the activity of the reporter plasmid co-transfected with an empty plasmid. To remove background in P19 cells, the normalized activity of the luciferase reporter plasmid co-transfected with an empty plasmid was subtracted from its activity in the presence of transcription factors. The activation of *Atp2a1* promoter by Gli2 was normalized to 1 and expressed as a percentage of MyoD alone.

### 4.3.15 Statistical analysis

To determine statistical significance between mean values of two groups, ANOVA followed by post-hoc Tukey HSD test was performed using XLSTAT software (Addinsoft, NY), (*, $p<0.05$; **, $p<0.01$).

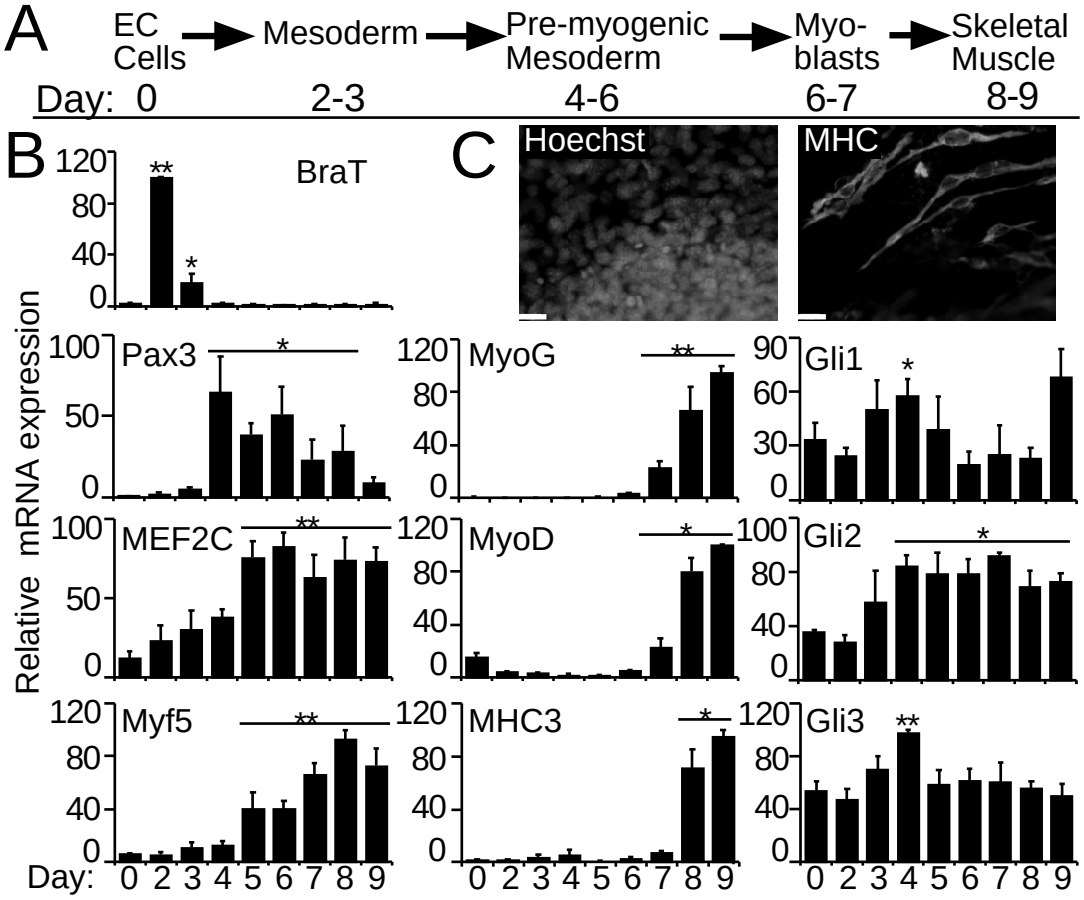
## 4.4 Results

### 4.4.1 Gli transcription factors are expressed during P19 EC skeletal myogenesis

Myogenic differentiation of P19 cells follows similar stages to that of embryonic myogenesis, and is schematically presented in Fig. 4.1A. QPCR analysis of transcript levels (Fig. 1B) demonstrated an induction of *BraT* mRNA starting from day 2, indicative of mesoderm induction (Fig. 4.1B). Elevated expression of *Pax3* transcripts during days 4-8 and of *MyoG* and *MyoD* transcripts on days 7-9 of P19 differentiation (Fig. 1B) indicated the specification and commitment of skeletal myogenesis, respectively, whereas increased transcript levels from the muscle structural gene, *MHC3* (Fig. 4.1B, panel MHC3), indicated differentiation into skeletal muscle. The presence of skeletal muscle was also observed by indirect immunofluorescence using MHC-specific antibodies (Fig. 4.1C). *MEF2C* and *Myf5* transcripts were elevated during days 5-9 of DMSO-induced differentiation of P19 cells (Fig. 1B, panel MEF2C). The relatively early expression of *MEF2C* could be attributed to cardiomyogenesis in P19 cells (39), and early expression of *Myf5* could be indicative of brown adipogenesis (66). This data correlates with previous reports and supports the presence of embryonic stages of myogenesis (39, 43, 67).

**Figure 4.1. Gli transcription factors are expressed during P19 DMSO-induced skeletal myogenesis.**

P19 cells were differentiated in the presence of 1% DMSO. **(A):** Schematic representation of skeletal myogenesis in P19 cells according to (39). **(B):** QPCR analysis of the expression of indicated genes at the time shown. Error bars represent +/- SEM, n=3, *$p<0.05$ and **$p<0.01$ relative to day 0. **(C):** Day 9 differentiated P19 cells were examined by immunofluorescence for MHC expression using MF20 antibodies. Nuclei were stained with Hoechst dye. Scale bar is 30 μm.


A
EC Cells
Mesoderm
Pre-myogenic Mesoderm
Myo-blasts
Skeletal Muscle
Day: 0
2-3
4-6
6-7
8-9
B
BraT
C
Hoechst
MHC
Relative mRNA expression
Pax3
MyoG
Gli1
MEF2C
MyoD
Gli2
Myf5
MHC3
Gli3
Day: 0 2 3 4 5 6 7 8 9

In terms of Hh signalling, the expression of *Gli1*, which is considered to be an indicator of active Hh signalling (68-70), was similar in undifferentiated P19 and mouse ES cells (Appendix I Fig. S1), supporting a role for Hh signalling in maintenance and proliferation of stem cells (71-74). During DMSO-induced differentiation of P19 cells, *Gli1* mRNA was significantly ($p<0.05$) elevated only on day 4 (Fig. 4.1B). The expression of *Gli3* mRNA was also slightly elevated only on day 4 of differentiation (Fig. 4.1B). In contrast, *Gli2* expression was significantly ($p<0.05$) increased during days 4-9 of P19 myogenesis (Fig. 4.1B). Overall, the level of *Gli2* mRNA increases as of pre-myogenic mesoderm formation, correlating with upregulation of *Pax3*, and is maintained throughout myogenesis. This agrees with the *in vivo* expression of Gli2 (6), thus making Gli2 an ideal candidate for studying the role of Hh signalling in the regulation of MyoD expression in P19 EC cells.

### 4.4.2 Gli2 regulates MyoD expression in P19 cells

To study the effect of Gli2 on MyoD expression during early skeletal myogenesis *in vitro*, we examined cells that stably overexpressed Gli2 in P19 cells (Fig. 4.2A-B). Stable overexpression of *Gli2* (Fig. 4.2B) led to an enhancement in skeletal muscle cell formation in P19[Gli2] cells when compared to the control cell line (Fig. 4.2A and Appendix I Fig. S2), after aggregation in the presence of DMSO. This was confirmed by QPCR analysis, which showed increased levels of *MEF2C*, *MyoD*, *Myf5*, *MyoG* and *MHC3* mRNA in P19[Gli2] cells on day 9 of differentiation (Fig. 4.2B). This supports previous publications, where Gli2 was shown to induce skeletal myogenesis in the absence of DMSO (46, 75). Therefore, Gli2 upregulates expression of *MyoD* and *MEF2C* while enhancing skeletal myogenesis in the presence of DMSO (summarized in Table 4.1).

**Figure 4.2. Gli2 regulates MyoD and MEF2C expression during skeletal myogenesis in P19 cells.**

P19[Gli2] or P19[Gli/EnR] and their respective control cell lines were differentiated in the presence of 1% DMSO. **(A, C):** Day 9 P19[Gli2] or P19[Gli/EnR] and their respective control cell lines were examined by immunofluorescence for MHC expression. Nuclei were stained with Hoechst dye. Scale bar is 30 μm. **(B, D):** QPCR analysis of the expression of indicated genes in P19[Gli2] (B) or [Gli/EnR] (D) and their respective control cell lines at the time shown. Error bars represent +/- SEM from two clonal populations and two biological replicas (n=4), *$p<0.05$ and **$p<0.01$.

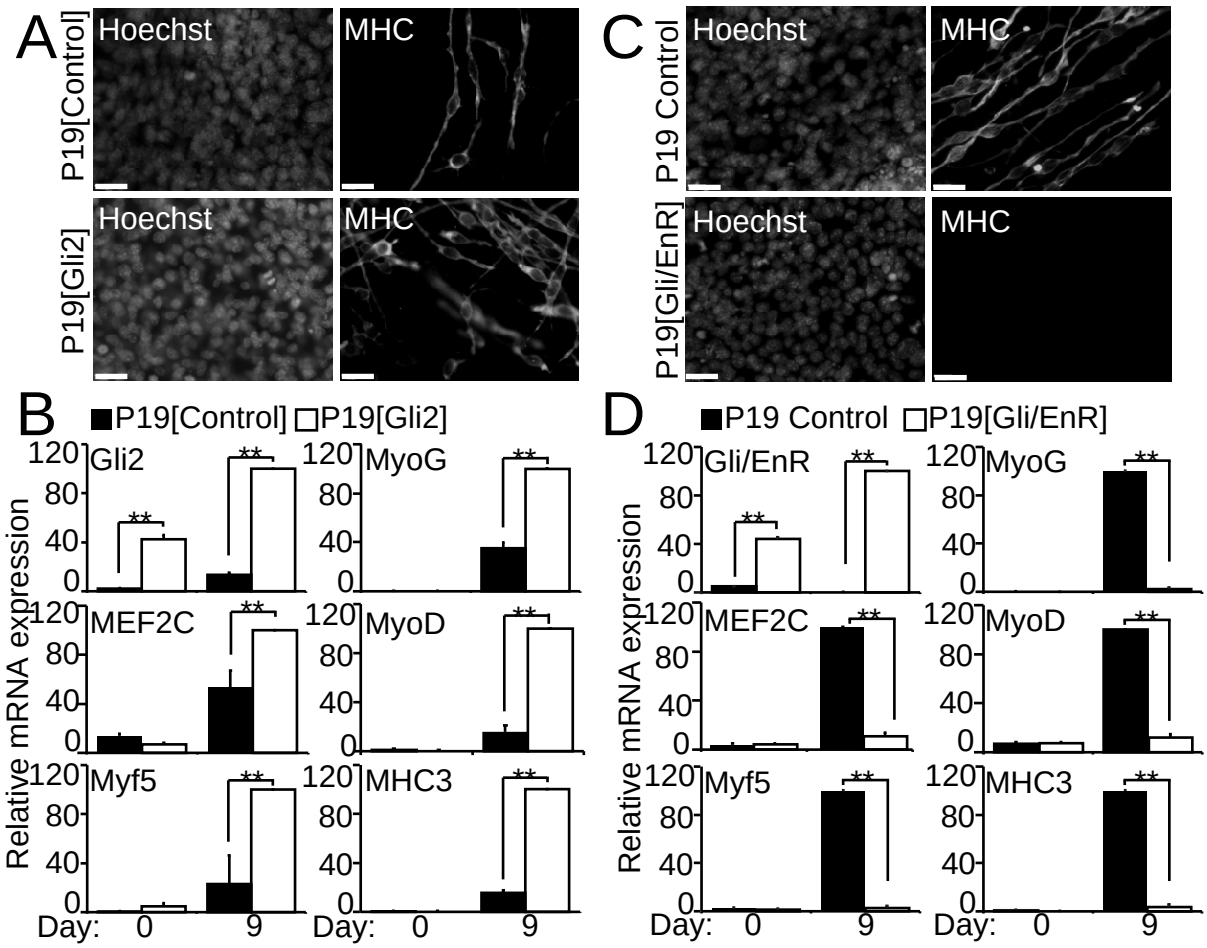

A
P19[Control]
Hoechst
MHC
P19[Gli2]
Hoechst
MHC
C
P19 Control
Hoechst
MHC
P19[Gli/EnR]
Hoechst
MHC
B
P19[Control] P19[Gli2]
Gli2
MyoG
MEF2C
MyoD
Myf5
MHC3
Relative mRNA expression
120
80
40
0
**
Day: 0 9
D
P19 Control P19[Gli/EnR]
Gli/EnR
MyoG
MEF2C
MyoD
Myf5
MHC3
Relative mRNA expression
Day: 0 9

**Table 4.1. Summary of gene expression changes in P19 cell lines treated with or without DMSO.**

Cell lines indicated on the left were aggregated under the conditions described in Materials and Methods and the induction of muscle marker gene expression was monitored. (+++ = high expression; ++ = elevated expression; + = normal expression; +/- = partial expression, - = not expressed or basal level of expression; N.D. = not determined).

| Cell line | Treat-ment | BraT | Gli2 | MEF2C | Pax3 | MyoD | Myf5 | $MHC^{+ve}$ myocytes | Reference |
|---|---|---|---|---|---|---|---|---|---|
| P19 | - | + | - | - | - | - | - | - | (39) |
| P19 | DMSO | + | + | + | + | + | + | <5% | Fig. 4.1 & (39) |
| P19[Gli2] | - | N.D. | +++ | + | + | + | + | <5% | (46, 75, 84) |
| P19[Gli2] | DMSO | + | +++ | ++ | ++ | ++ | ++ | ~9% | Figs. 4.2, Appendix I Fig. S2 and (63) |
| P19[MEF2C-TAP] | DMSO | + | ++ | +++ | N.D. | ++ | N.D. | ~5% | Appendix I Fig. S6 and (36, 63, 85) |
| P19[Gli/EnR] | DMSO | + | + | - | +/- | - | - | - | Fig. 4.2 & (46, 75) |

Since all Gli factors bind the same DNA sequence and have overlapping functions (6, 68, 76, 77), we used a dominant negative Gli2/Engrailed (Gli/EnR) mutant fusion protein, repressing all Gli2-bound regulatory elements, to evaluate the role of Gli factors during myogenesis (46, 63, 78, 79). This approach has been used successfully both *in vitro* and *in vivo* (44, 45, 67, 78, 80-82). Furthermore, overexpression of the Engrailed repressor domain does not interfere with differentiation *in vitro* (83). Stable overexpression of *Gli/EnR* (Fig. 4.2D) abrogated DMSO-induced skeletal myogenesis in P19 cells (Fig. 4.2C), in accordance with previous studies (46, 75). There was also a concomitant robust downregulation of *MEF2C*, *MyoD*, *Myf5*, *MyoG* and *MHC3* expression on day 9 of P19[Gli/EnR] differentiation (Fig. 4.2D). The effect of Gli/EnR was not due to global gene repression since P19[Gli/EnR] cells still expressed the mesoderm marker *BraT* (46) and differentiated into cardiomyocytes, neurons and astrocytes, albeit with decreased efficiency (63, 79, Chapters 2 & 3). Therefore, we confirm initial findings that Gli/EnR downregulated *Myf5* and *MyoD*, inhibiting myogenesis (46, 75), and extend them to show inhibition of *MEF2C* and *MyoG* transcripts (summarized in Table 4.1).

### 4.5 Gli2 associates with *MyoD* gene elements and activates the *MyoD* promoter

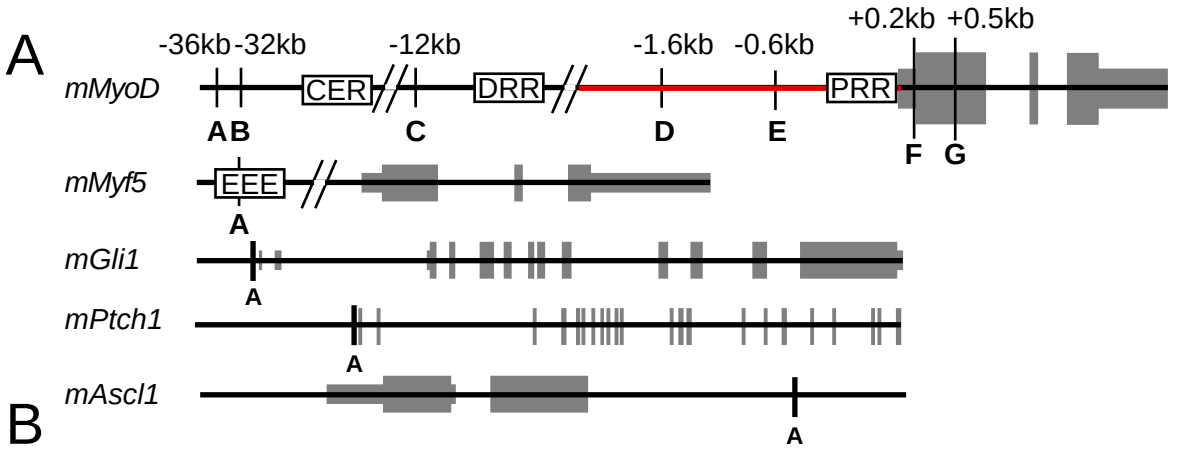
To determine if Gli2 directly regulates MyoD expression during skeletal myogenesis, we performed chromatin immunoprecipitation (ChIP) experiments. *In silico* analysis of the *MyoD* gene (±100 kb) revealed 7 conserved consensus Gli binding sites (*MyoD* A-G, Fig. 4.3A-B & Appendix I Fig. S3), suggesting that MyoD could be a direct target of a Gli transcription factor. Since the highest expression of *Gli2* mRNA and protein occurs on day 4 of P19[Gli2] differentiation (63, 79, Chapters 2 & 3), and transcription factors are known to bind target genomic DNA before initiating transcription (47), we performed ChIP analysis

with Gli2-specific antibodies on day 4 differentiating P19[Gli2] cells. There was a statistically significant enrichment in chromatin fragments corresponding to *MyoD* sites A-B and D-G (Fig. 4.3C). *MyoD* site C appeared not to be bound by Gli2 (Fig. 4.3C), indicating specificity of the ChIP assay with Gli2 antibodies. The *Gli1* and *Ptch1* promoters and *Myf5* EEE region were used as positive controls and one of the *Ascl1* gene element was used as a negative control (27, 79, 86, 87). Thus, Gli2 associates with *MyoD* gene regulatory regions in differentiating P19 cells (summarized in Table 4.2).

To determine if the interaction of Gli2 with the *MyoD* proximal promoter (Fig. 4.3A, red line) was functionally relevant, we performed reporter assays in C3H10T1/2 cells with a *-2.2 kb MyoD* proximal promoter driving Firefly luciferase expression (Fig. 4.3D). Gli2 activated the *MyoD* proximal promoter in a concentration-dependent manner and to a similar extent as MyoD or MEF2C (Fig. 4.3D). Therefore, the association of Gli2 with the *MyoD* proximal promoter leads to *in vitro* promoter activation.

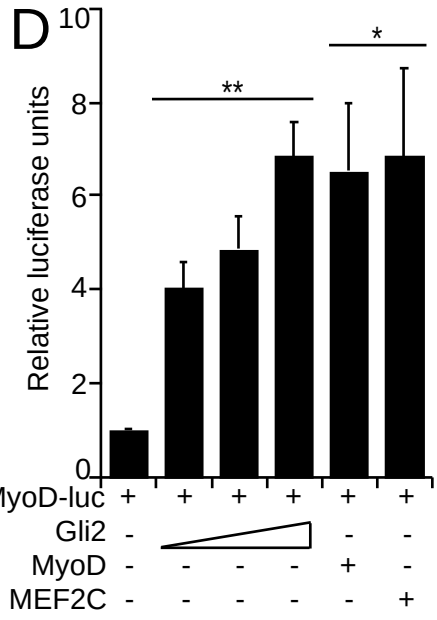
**Figure 4.3. Gli2 associates with *MyoD* gene elements during skeletal myogenesis and activates the *MyoD* promoter.**

**(A):** Custom tracks of murine *MyoD*, *Myf5*, *Gli1*, *Ptch1* and *Ascl1* genes. Letters designate conserved Gli binding sites (GBS), their genomic positions are listed relative to the transcriptional start site (TSS). Grey boxes designate exons. PRR (proximal regulatory region, RR) is located at -0.3 kb, DRR (distal RR) at -5 kb and CER (core enhancer region) at -20 kb relative to MyoD TSS. **(B):** Sequence conservation in species indicated for *MyoD* A-G sites from (A). The sequence of the GBS is marked in bold. **(C):** ChIP analysis of Gli2-bound *MyoD*, *Myf5*, *Gli1*, *Ptch1* and *Ascl1* genes on day 4 of P19[Gli2] cellular differentiation. Black bars designate genomic regions immunoprecipitated with Gli2-specific antibodies, and white bars designate genomic regions precipitated with IgG-nonspecific antibodies. Percent chromatin input was calculated using QPCR analysis and normalized to IgG. Error bars represent +/- SEM (n=3). **(D):** Gli2 activates the *MyoD* promoter in a concentration-dependent manner. The *-2.2 kb MyoD* reporter construct (MyoD-luc, depicted as a red line in A) was co-transfected in C3H10T1/2 cells with Gli2 in ratios 2:1, 4:1 and 6:1 relative to MyoD-luc, or with MyoD-Flag or MEF2C-Flag in ratio 4:1 relative to MyoD-luc. Luciferase activity was normalized to Renilla and expressed as fold change over MyoD-luc co-transfected with an empty plasmid. Error bars represent +/- SEM (n=3). *$p<0.05$, **$p<0.01$.




B

| MyoD site | Species | Sequence | % GBS conserved |
|---|---|---|---|
| A | *H. sapiens* | TTG**GGAACCCCCAGT**GGG | |
| | *M. musculus* | TTG**GGAACCCCCAGT**GGG | |
| | *R. norvegicus* | TTG**GGAACCCCCAGT**GGG | 91.7 |
| | *E. caballus* | TTG**GGAACCCCCAGA**GGG | |
| | *C. lupus f.* | TTG**GGAACCCCCAGT**GGG | |
| B | *H. sapiens* | GAG**TCTGGGAGGGCT**TGG | 91.7 |
| | *M. musculus* | AAG**TCTGGGTGGGCT**TAT | |
| C | *H. sapiens* | CCC**ACCCCACCCACT**GCT | 100 |
| | *M. musculus* | CCC**ACCCCACCCACT**GCT | |
| D | *H. sapiens* | GAG**TCTTGGTTTTGT**TCA | 66.7 |
| | *M. musculus* | GGG**CCTTGGTTGCGC**TTA | |
| E | *H. sapiens* | TAG**C--TGGGTTTCT**AAG | 75 |
| | *M. musculus* | CAG**CGCTGGGGTTCT**AAG | |
| F | *H. sapiens* | CCG**CTTGGGTTGGG-**-CG | 83.3 |
| | *M. musculus* | CGG**CTTGGGTTGAGG**CTG | |
| G | *H. sapiens* | GCA**AGACCACCAACG**CCG | 100 |
| | *M. musculus* | GCA**AGACCACCAACG**CTG | |



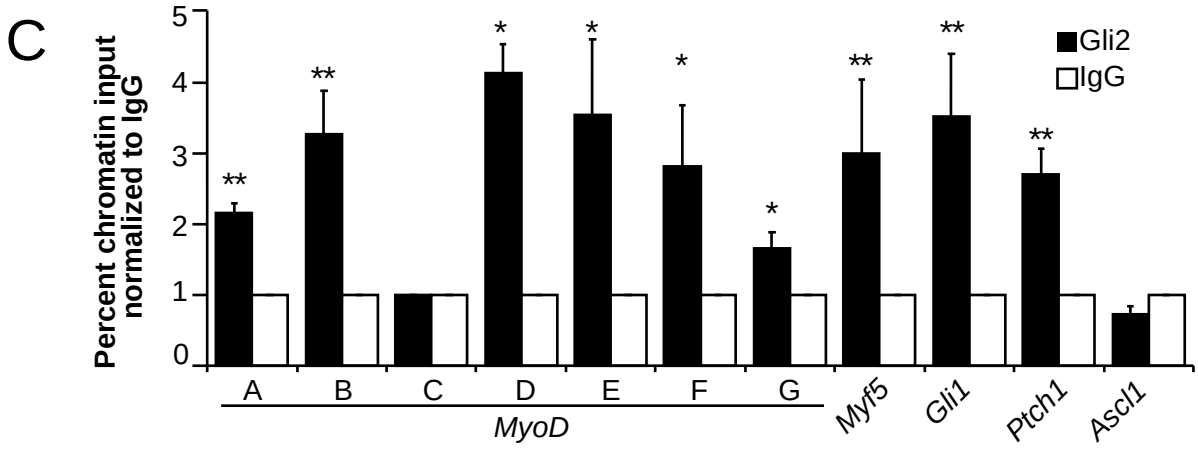



**Table 4.2. Detailed analysis of genomic regions studied in ChIP-QPCR experiments.**

Chr = chromosome, BS = binding site.

| Target gene | Position of BS in mm9 genome | Strand Orien-tation | Forward primer | Reverse Primer | Binding by MyoD/ MyoG* | Binding by Gli2[§] |
|---|---|---|---|---|---|---|
| *MyoD* A | Chr7:53595554-53595566 | + | AAGGAGGGGGAGGGAATAAT | TTAAACGCCTTCCTCGATTG | MyoD/ MyoG[#] | + |
| *MyoD* B | Chr7:53599767-53599779 | + | CAGAAGTCTGGGTGGGCTTA | TCAGCCTCATAACCCAAAGG | - | + |
| *MyoD* C | Chr7: 53619900-53619912 | + | CTGCCTGTGCTGCCTCAT | GAAGCTCTCAGCAAGCAGTG | MyoD/ MyoG | - |
| *MyoD* D | Chr7: 53630159-53630171 | + | CTATGTGCATAGGGCCTTGG | CACCCAGGAACCTCTTTTGA | - | + |
| *MyoD* E | Chr7: 53631228-53631240 | + | CCTCCCTCTTTTCCTTGGAC | AAGCCTGGCACAAATGAATC | - | + |
| *MyoD* F | Chr7: 53632010-53632022 | + | GACAGGGAGGAGGGGTAGAG | TGCTGTCTCAAAGGAGCAGA | MyoD/ MyoG | + |
| *MyoD* G | Chr7: 53632353-53632365 | + | GTGCAAGCGCAAGACCAC | CAGGATCTCCACCTTGGGTA | MyoG only | + |
| *Myf5* A | Chr10: 106928984-106928993 | - | ACTGGACCAAAACAGCAAAGC | CTCTGCTTTCTTCCCCACTG | - | + |
| *Ptch1* A | Chr13: 63667821-63667833 | - | TATTGCATGCGAGAGGGTTG | GGAGGGCAGAAATTACTCAGC | MyoG only | + |
| *Gli1* A | Chr10: 126778677-126778689 | - | GCACCCCCTCTCTAGCTTCTATC | GGACCACCCGCGAGAAGCGCAAACT | - | + |
| *Ascl1* A | Chr10: 86936603-86936615 | - | CCTAAGATCAATGGGCCAAA | CCCACCCAACTGTCCTAGAG | - | - |

* Data was obtained from MyoD and MyoG ChIP-seq peaks in C2C12 cells from ENCODE/Calbiotech dataset as described in (51);

[§] Based on Fig. 4.3C

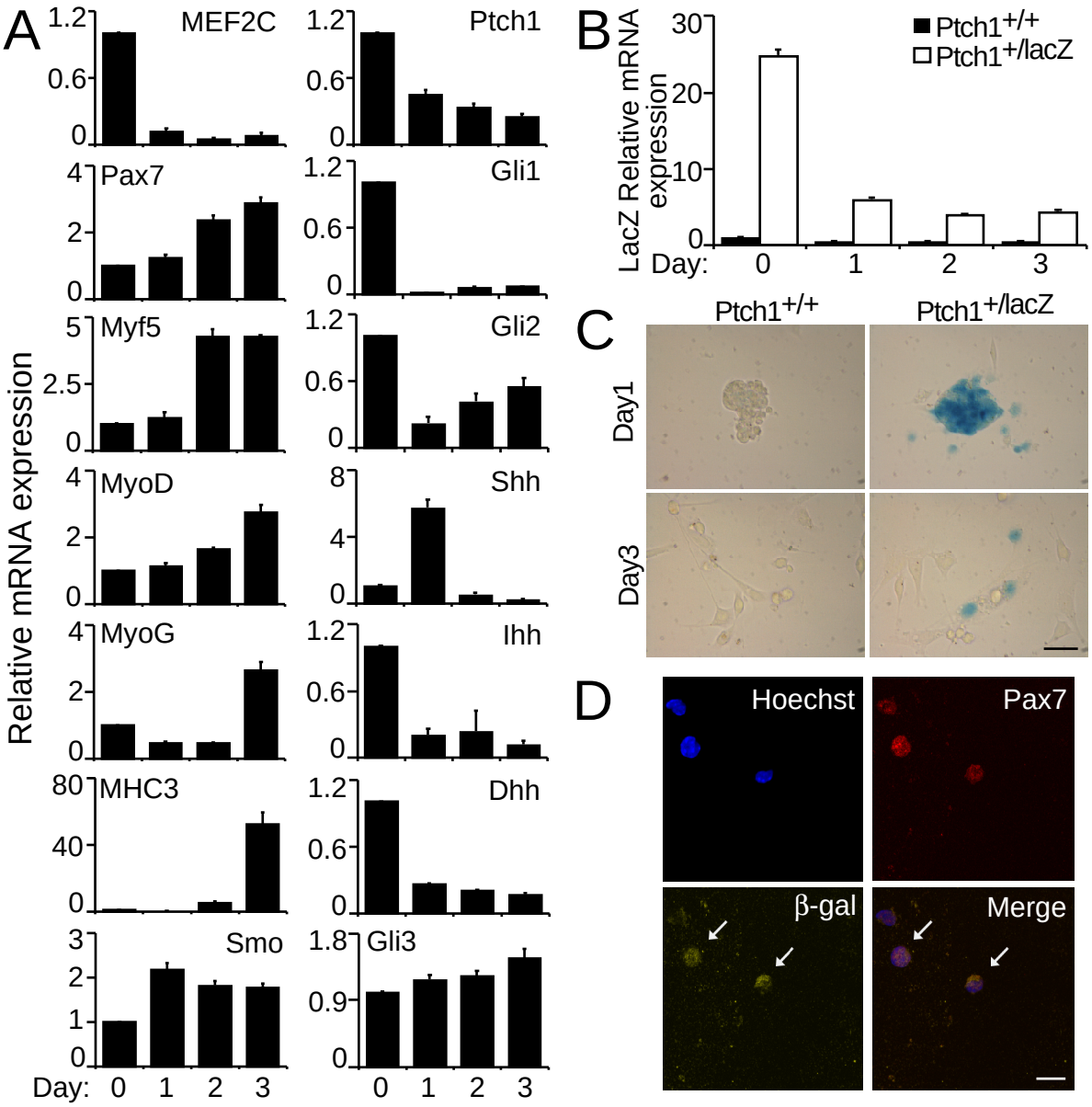
[#] MyoG and MyoD ChIP-Seq peaks were observed within 1-2 kb from Gli BS, respectively.

### 4.5.1 Members of the Hh signalling pathway are expressed during muscle satellite cell activation *in vitro*

We had previously shown that signalling pathways regulating muscle commitment in P19 cells also regulate satellite cell activation (36). To determine if the Hh signalling pathway is active during SC activation *in vitro*, we analyzed expression of the Hh signalling components in a three-day culture of freshly isolated muscle SCs. Under these conditions, both the activation of SCs and their early differentiation without multinucleated myotube formation could be observed (36), shown by the upregulation of the satellite cell marker *Pax7*, the myoblast markers *Myf5* and *MyoD*, and the differentiation markers *MyoG* and *MHC3* (Fig. 4.4A). Transcripts for the markers of active Hh signalling *Ptch1* and *Gli1* (56, 70, 88), as well as *Gli2*, *MEF2C* and the Hh ligands *Ihh* and *Dhh* were downregulated after 1 day in monolayer culture (Fig. 4.4A). The expression of *Shh* peaked on day 1, whereas *Smo* and *Gli3* expression remained relatively constant (Fig. 4.4A). Although *Gli1* was downregulated after culturing the satellite cells, it is important to note that the levels of *Gli1* remaining are similar to the levels found in C3H10T1/2 cells treated with MyoD (Appendix I Fig. S1).

**Figure 4.4. Muscle satellite cells respond to Hh signalling and express members of the Hh signalling pathway during muscle satellite cell activation *in vitro*.**

Freshly isolated mouse SCs were cultured in the absence of growth factors for 3 days. **(A):** QPCR analysis of the expression of indicated genes in SCs at the time shown. Error bars represent +/- SEM (n=3). **(B):** QPCR analysis of *LacZ* mRNA expression in SCs isolated from $Ptch1^{+/+}$ or $Ptch1^{+/LacZ}$ mice at the time shown. Error bars represent +/- SEM (n=4). **(C):** Day 1 and day 3 cultured SCs from $Ptch1^{+/+}$ or $Ptch1^{+/LacZ}$ mice were assayed for β-gal activity. Scale bar is 30 μm. **(D):** Day 1 cultured SCs from $Ptch1^{+/LacZ}$ mice were analyzed by immunofluorescence for Pax7 (red) and β-gal (yellow) expression using specific antibodies. Nuclei were stained with Hoechst dye (blue). Arrows indicate $\beta\text{-gal}^{+}$ cells. Scale bar is 20 μm.


A
MEF2C
Ptch1
Pax7
Gli1
Myf5
Gli2
MyoD
Shh
MyoG
Ihh
MHC3
Dhh
Smo
Gli3
Relative mRNA expression
Day: 0 1 2 3
B
Ptch1+/+
Ptch1+/lacZ
LacZ Relative mRNA expression
Day: 0 1 2 3
C
Ptch1+/+
Ptch1+/lacZ
Day1
Day3
D
Hoechst
Pax7
β-gal
Merge

To confirm that Hh signalling occurs during SC activation, we analyzed the expression of β-gal in SCs isolated from heterozygous Ptch1 reporter ($Ptch1^{+/LacZ}$) mice. The expression of LacZ in these animals is under the control of the Ptch1 promoter and has a nuclear localization signal (56). During SC activation *in vitro*, the expression of LacZ mRNA (Fig. 4.4B) closely resembled that of Ptch1 in wild type (wt) cells (Fig. 4.4A, panel Ptch1). This was confirmed by β-gal staining, where there was a higher percentage of $\beta\text{-gal}^+$ cells on day 1 as compared to day 3 during satellite cell activation in vitro. Notably, there was no LacZ expression or activity detected in wt $Ptch1^{+/+}$ cells (Fig. 4.4B-C). Indirect immunofluorescence analysis revealed that $\beta\text{-gal}^+$ SCs isolated from $Ptch1^{+/LacZ}$ mice were also $Pax7^+$ (Fig. 4.4D). Thus, muscle satellite cells express and respond to Hh signalling during SC activation *in vitro*.

### 4.5.2 Modulation of Hh signalling perturbs MyoD expression during muscle satellite cell activation *in vitro*

To study the effect of Hh signalling on MyoD expression during SC activation *in vitro*, we inhibited Hh signalling while culturing freshly isolated murine SCs for three days. Hh inhibitors included cyclopamine or KAAD-cyclopamine, which are pharmaceutical reagents that specifically bind to the Smo receptor leading to inhibition of Hh signalling (89), and inhibitory Shh-specific antibodies (90, 91) (Fig. 4.5). SB203580 (SB), a MAP (mitogen-activated protein) kinase inhibitor, was used as a positive control (36, 92). The QPCR analysis revealed that *Gli2* expression was not statistically significantly downregulated by Hh signalling inhibitors (Fig. 4.5), however, the primary effect of Hh signalling inhibition is the repression of Gli2 protein function, as opposed to mRNA expression (reviewed in (22)). In accordance, the expression of *Gli1* was significantly downregulated in the presence of

cyclopamine, KAAD-cyclopamine and Shh-specific antibodies (Appendix I Fig. S4), confirming inhibition of Hh signalling. There was a modest decrease in *Myf5* and *Pax7* expression by cyclopamine and Shh-specific antibodies that did not reach statistical significance (Fig. 4.5). Importantly, expression of *MEF2C*, *MyoD* and *MHC3* was significantly downregulated in the presence of Hh signalling inhibitors, similar to SB (Fig. 4.5). The upregulation of *Pax7* and *Myf5* expression by SB is in accordance with previous reports (36, 92). Therefore, inhibition of Hh signalling by two different approaches resulted in downregulation of *MyoD* and *MHC3* expression during culture of satellite cells, indicating a reduction in satellite cell activation and differentiation.

### 4.5.3 Hh signalling regulates MyoD activity

To test if Hh signalling is important for MyoD transcriptional activity, we examined the effect of Hh signalling inhibition on myogenic conversion assays, using Hh-responsive C3H10T1/2 fibroblasts (93). Exogenous MyoD successfully converted C3H10T1/2 fibroblasts into muscle as assessed by upregulation of endogenous *MHC3*, *MEF2C* (Fig. 4.6A) and *MyoG* (Appendix I Fig. S5) transcripts, in accordance with previous reports (20). The expression of *Gli1* was elevated in MyoD-transfected fibroblasts (Fig. 4.6A & Appendix I Fig. S1), indicating that MyoD expression can activate Hh signalling. Subsequent inhibition of Hh signalling with KAAD-cyclopamine or dominant-negative Gli2 (Gli/EnR), resulted in a failure of MyoD to efficiently upregulate the transcription of *MHC3*, *MEF2C* and *MyoG* (Fig. 4.6A & Appendix I Fig. S5). Since MEF2 factors were shown to be important for MyoD activity in myogenic conversion assays (94), we sought to determine whether exogenous MEF2C could rescue the observed phenotype.

**Figure 4.5. Hh signalling regulates MyoD expression during muscle satellite cell activation *in vitro*.**

QPCR analysis of indicated genes on day 0 (untreated) and day 3 of cultured mouse SCs in the presence or absence of cyclopamine (cyclop), KAAD-cyclopamine (KAAD) (grey bars), SB203580 (SB) (white bars) and their respective vehicles (MeOH; methanol) or in the presence of Shh-specific (striped bars) or IgG non-specific antibodies for 3 days. Data from treatment with a specific reagent and its respective vehicle is separated by dashed line and expressed as a percentage of day 3 levels. Error bars represent +/- SEM (n=3). *$p<0.05$, **$p<0.01$.

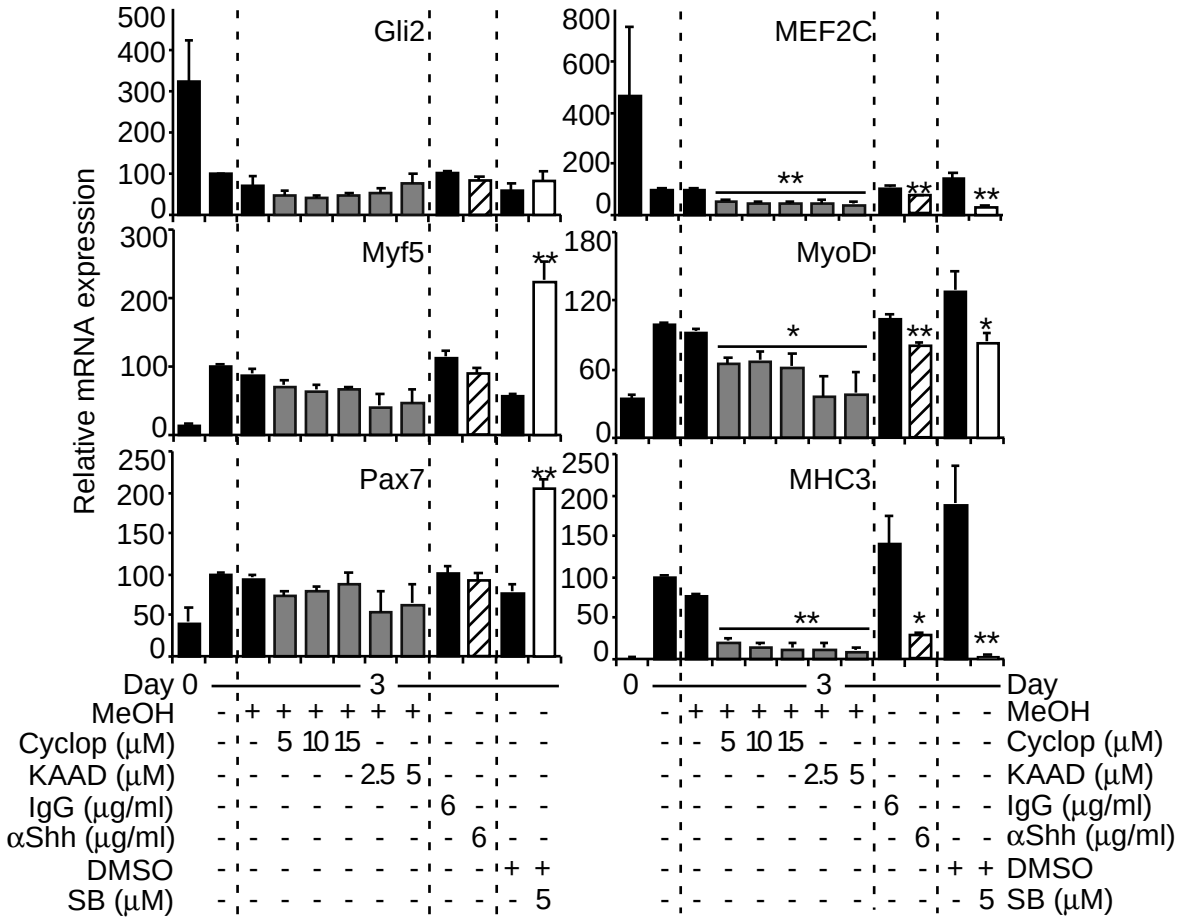
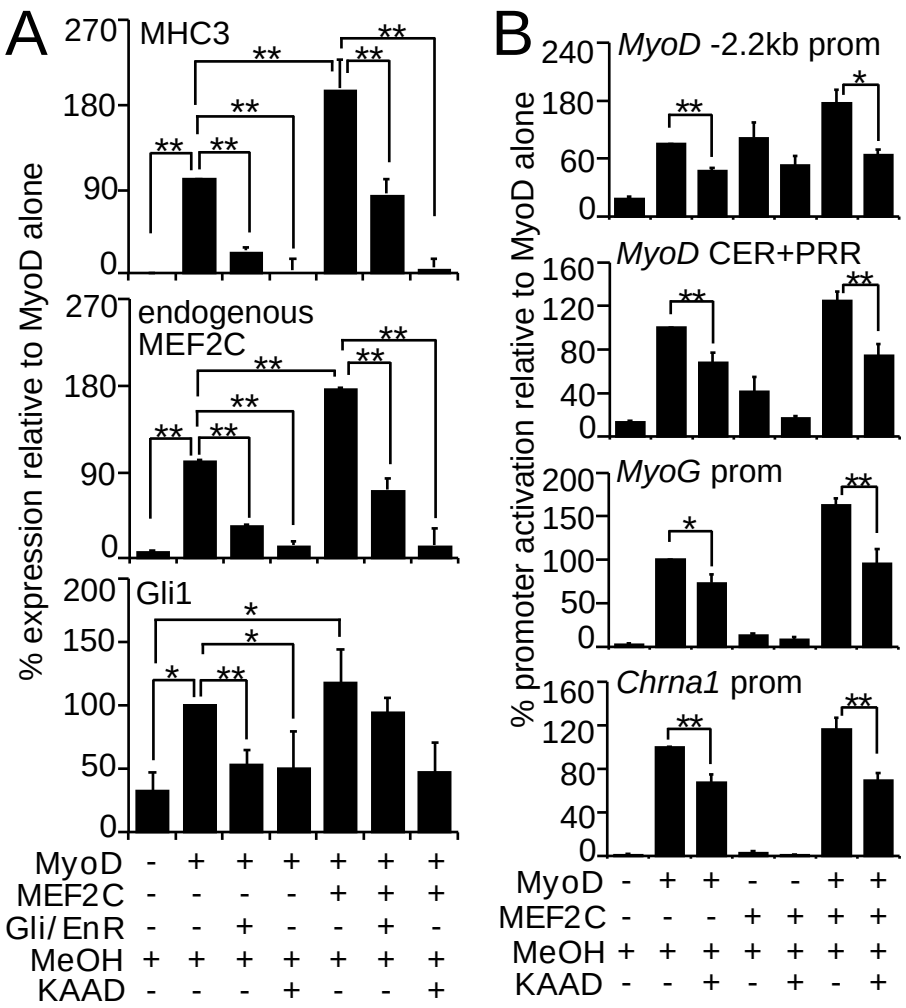

Gli2
MEF2C
Myf5
MyoD
Pax7
MHC3
Relative mRNA expression
Day 0 3
MeOH
Cyclop (μM)
KAAD (μM)
IgG (μg/ml)
αShh (μg/ml)
DMSO
SB (μM)

**Figure 4.6. Hh signalling modulates MyoD activity.**

C3H10T1/2 fibroblasts were transfected with MyoD, Gli2, Gli/EnR and/or MEF2C-expressing plasmids and induced to undergo myogenic conversion. **(A-B):** Inhibition of Hh signalling in C3H10T1/2 cells reduces transcriptional activity of MyoD with or without MEF2C. **(A):** QPCR analysis of *MHC3*, endogenous *MEF2C* and *Gli1* mRNA in C3H10T1/2 fibroblasts after 48h incubation in starvation media with or without KAAD-cyclopamine (KAAD). Data was calculated as fold change over C3H10T1/2 fibroblasts transfected with an empty vector, as a percentage of MyoD alone. Error bars represent +/- SEM (n=4). *$p<0.05$, **$p<0.01$. **(B):** Reporter analysis of C3H10T1/2 cells co-transfected with -2.2 kb *MyoD* promoter, *MyoD* CER+PRR, *MyoG* or *Chrna1* promoter reporter plasmids and MyoD and/or MEF2C expressing plasmids in ratios 3:1 to reporter construct. Transfected cells were cultured in starvation media in the presence of KAAD-cyclopamine or vehicle (MeOH). Luciferase activity was normalized to Renilla and expressed as fold change over reporter construct co-transfected with an empty plasmid, as a percentage of MyoD alone. Error bars represent +/- SEM (n=5). *$p<0.05$, **$p<0.01$


A
MHC3
endogenous MEF2C
Gli1
% expression relative to MyoD alone
MyoD
MEF2C
Gli/EnR
MeOH
KAAD
B
MyoD -2.2kb prom
MyoD CER+PRR
MyoG prom
Chrna1 prom
% promoter activation relative to MyoD alone

Co-transfection with MEF2C enhanced the ability of MyoD to upregulate *MHC3* (Fig. 4.6A), in agreement with previous reports (95, 96). However, there was a significant reduction in the ability of the MyoD-MEF2C complex to convert C3H10T1/2 fibroblasts into muscle in the presence of KAAD-cyclopamine or Gli/EnR (Fig. 4.6A, panels MHC3 & MEF2C). Therefore, MyoD activates and requires Hh signalling for conversion of fibroblasts into muscle.

Exogenous Gli2 did not affect MyoD-induced expression of *MHC3* and slightly downregulated the expression of *MyoG* (Appendix I Fig. S5), suggesting that Gli2 is not rate-limiting and may squelch MyoD activity at higher concentrations (97). Nevertheless, cells co-transfected with Gli2 and MyoD and treated with KAAD-cyclopamine exhibited reduced *MHC3* and *MyoG* expression, confirming the importance of Hh signalling for MyoD activity (Appendix I Fig. S5).

To test if inhibition of Hh signalling alters the ability of MyoD to activate exogenous target promoters during myogenic conversion assays, we analyzed the activation of known MyoD targets, including the *MyoD* CER/PRR (core enhancer region/proximal regulatory region), *MyoD -2.2 kb*, *MyoG* and *Chrna1* promoters, in reporter assays in C3H10T1/2 cells, with or without KAAD-cyclopamine. In the presence of KAAD-cyclopamine there was a 30-35% decrease in MyoD-mediated activation of promoters as compared to the vehicle control (Fig. 4.6B). Although there was not a statistically significant decrease in MEF2C transactivation in the presence of KAAD-cyclopamine, there was a 30-45% decrease in MyoD/MEF2C-mediated promoter activation with KAAD-cyclopamine (Fig. 4.6B).

Therefore, we demonstrate for the first time that endogenous Hh signalling regulates MyoD function on endogenous and exogenous promoters during C3H10T1/2 fibroblast conversion assays.

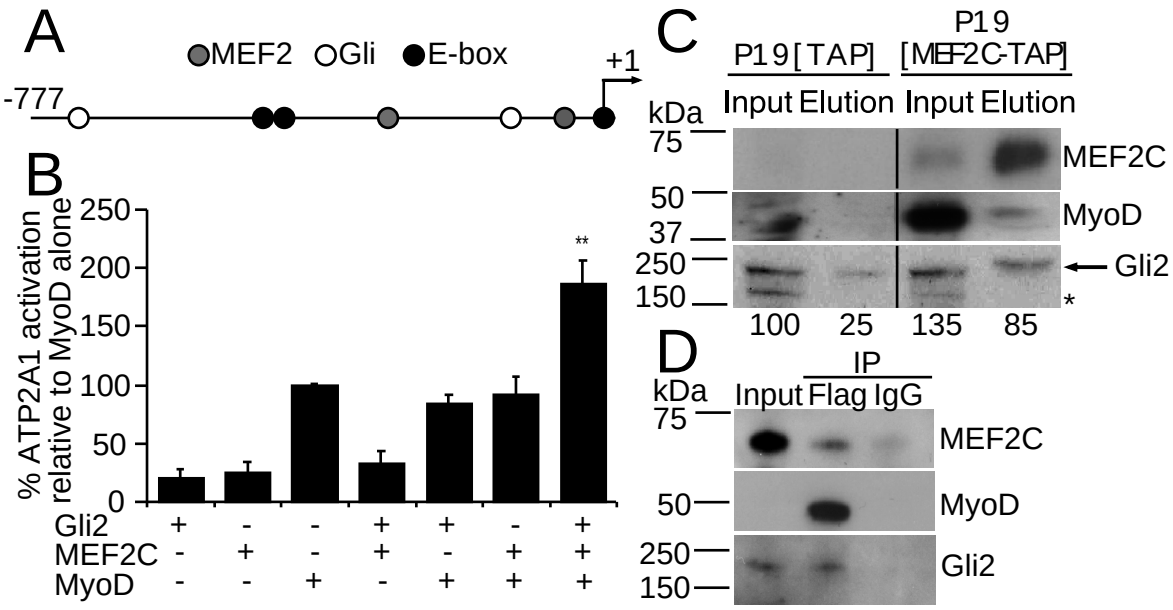
### 4.5.4 Gli2, MEF2C and MyoD form a protein complex, which enhances MyoD activity

We have recently reported that Gli2 and MEF2C form a protein complex (63, Chapter 3) and it was previously known that MyoD and MEF2C proteins interact during skeletal myogenesis (95). To test if Gli2, MEF2C and MyoD form a protein complex, we first tested the ability of Gli2 and MEF2C to enhance MyoD activity on the *Atp2a1* promoter, which has 2 Gli, 2 MEF2 binding sites and 3 E-boxes (Fig. 4.7A) and drives the expression of SERCA1 ($Ca^{2+}$ ATPase) in fast-twitch skeletal muscle (98). When Gli2, MEF2C and MyoD were co-transfected together in P19 cells, they enhanced MyoD activity on the promoter, as compared to MyoD alone or in combination with Gli2 or MEF2C (Fig. 4.7B).

To test whether Gli2, MEF2C and MyoD physically associate in a protein complex during skeletal myogenesis, we used P19 cells stably overexpressing MEF2C-TAP, which show enhanced myogenesis (36) (Appendix I Fig. S6 & Table 4.1). We purified MEF2C-TAP protein from a nuclear protein extract of day 8 differentiating P19[MEF2C-TAP] cells, when MyoD and Gli2 are expressed. Subsequent western blot analysis showed co-purification of MEF2C-TAP with endogenous MyoD and Gli2 proteins (Fig. 4.7C). Although Gli2 protein was weakly present in the elution fraction of P19[TAP] control cells (Fig. 4.7C, panel Gli2), quantitative band-densitometry analysis identified a 3.4-fold increase in Gli2 band intensity in the eluted fraction of the P19[MEF2C-TAP] cultures, compared to the P19[TAP] cultures (Fig. 4.7C).

**Figure 4.7. Gli2, MEF2C and MyoD form a protein complex, which enhances MyoD activity.**

**(A):** Schematic representation of Atp2a1-luc reporter construct. Circles designate Gli (white), MEF2 (grey) and E-box (black) binding sites; -777 designates the beginning of the Atp2a1 promoter relative to transcriptional start site (+1). **(B):** Gli2 and MEF2C enhance MyoD-mediated activation of *Atp2a1* promoter. P19 cells were co-transfected with Atp2a1-luc reporter construct and MyoD-, Gli2- and/or MEF2C-expressing plasmids. Luciferase activity was measured after 24 h and normalized to Renilla. Fold change values were calculated as a percentage of MyoD alone. Error bars represent +/- SEM (n=7), **p<0.01. **(C):** MEF2C-TAP and endogenous Gli2 and MyoD co-immunoprecipitated from the nuclear fraction of day 8 differentiating P19[MEF2C-TAP], but not P19[TAP] cells. Numbers indicate density of the Gli2 band. Lanes were spliced out from the same autoradiogram as designated by vertical lines. Arrow designates Gli2 protein band, and asterisk denotes non-specific binding of the Gli2 antibodies (63, 64). **(D):** Exogenous Gli2, MEF2C-TAP and MyoD-Flag co-immunoprecipitated from transfected C3H10T1/2 cells. Transfected cells were induced to undergo myogenic conversion by incubation in starvation media for 24h and subjected to Flag-IP. The antibodies used for western blot assay are indicated on the right.


A
MEF2
Gli
E-box
+1
-777
B
% ATP2A1 activation relative to MyoD alone
250
200
150
100
50
0
**
Gli2 + - - + + - +
MEF2C - + - + - + +
MyoD - - + - + + +
C
P19 [TAP]
P19 [MEF2C-TAP]
Input Elution Input Elution
kDa
75
50
37
250
150
MEF2C
MyoD
Gli2
*
100 25 135 85
D
IP
kDa
Input Flag IgG
75
50
250
150
MEF2C
MyoD
Gli2

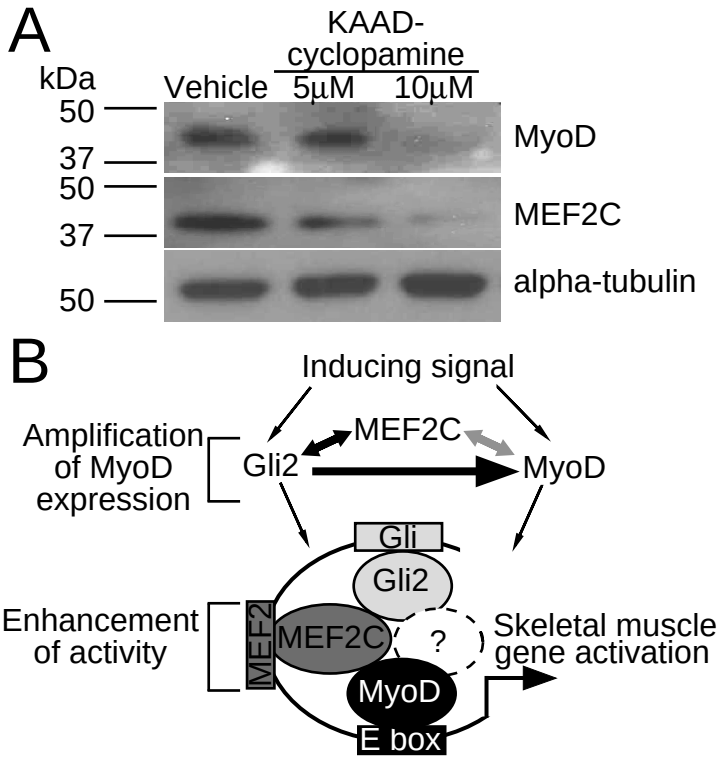
Moreover, we co-immunoprecipitated Gli2 and MEF2C with MyoD-Flag from C3H10T1/2 cells undergoing myogenic conversion (Fig. 4.7D), confirming the physical association of Gli2, MEF2C and MyoD proteins. Therefore, Gli2 and MEF2C enhance MyoD activity and physically form a protein complex with MyoD protein during skeletal myogenesis.

### 4.5.5 Hedgehog signalling regulates MyoD in an adult myoblast cell line

Since Hh signalling regulated MyoD expression (Figs. 4.2, 4.3 & 4.5) and protein function (Figs. 4.6 & 4.7), we sought to test if modulation of Hh signalling would result in attenuated levels of MyoD in Hh-responsive C2C12 myoblasts (93), a model of adult muscle regeneration (99). The incubation of proliferating C2C12 myoblasts in the presence of KAAD-cyclopamine resulted in downregulation of MEF2C and MyoD protein levels, with the biggest downregulation observed with 10 μM KAAD-cyclopamine (Fig. 4.8A). Therefore, endogenous Hh signalling regulates MyoD and MEF2C protein expression in C2C12 myoblasts, likely due to the inhibition of both MyoD expression and function.

**Figure 4.8. Hh signalling regulates MyoD expression and activity.**

**(A):** Hh signalling regulates MyoD and MEF2C protein expression in C2C12 myoblasts. C2C12 cells were grown for 24h and treated with KAAD-cyclopamine or vehicle (methanol). Total protein from treated cells was resolved and immunoblotted with MEF2C- or MyoD-specific antibodies, α-tubulin served as a loading control. **(B):** Proposed model for regulation by Hh signalling. Gli2, MEF2C and MyoD form a regulatory loop, where Gli2 induces MyoD expression (this study, designated by black thick arrow), Gli2 and MEF2C induce each other's expression ((63) and this study, black arrow) and MEF2C and MyoD regulate each other's expression ((100, 101), grey arrow). Gli2, MEF2C and MyoD form a protein complex, which participates in the activation of skeletal muscle-related gene promoters, where "?" represents possible uncharacterized protein(s) participating in Gli2/MEF2C/MyoD protein complex.


A
KAAD-cyclopamine
kDa
Vehicle
5μM
10μM
50
37
50
37
50
MyoD
MEF2C
alpha-tubulin
B
Inducing signal
Amplification of MyoD expression
MEF2C
Gli2
MyoD
Gli
Gli2
Enhancement of activity
MEF2
MEF2C
?
Skeletal muscle gene activation
MyoD
E box

## 4.6 Discussion

In this Chapter, we have shown that Hh signalling regulates MEF2C and MyoD expression during P19 skeletal myogenesis, in proliferating C2C12 myoblasts, and during mouse satellite cell activation *in vitro*. Moreover, Gli2 associates with *MyoD* gene elements in P19 cells and activates the *MyoD* promoter in transient transfection experiments. In addition to regulating the expression of MyoD, endogenous Hh signalling is important for efficient MyoD transcriptional activity in myogenic conversion assays and in promoter activity assays with MyoD target genes. Lastly, the protein complex consisting of Gli2, MEF2C and MyoD enhances MyoD activity on skeletal muscle-related promoter. Thus, we propose a model, where Gli2, MEF2C and MyoD participate in a regulatory loop by inducing and maintaining each other's expression, as well as forming a protein complex that activates gene expression during skeletal myogenesis (Fig. 4.8B).

We show for the first time that Gli2 associates with *MyoD* gene elements and activates the *MyoD* promoter *in vitro*. Our finding supports and extends previous publications, identifying Shh signalling as sufficient and essential for MyoD expression during early somitogenesis (23, 24) and expands previous work showing that Gli2 binds to the *Myf5* epaxial enhancer (27). Since exogenous Shh cannot rescue the expression of MyoD in presomitic mesoderm explanted from $Myf5^{-/-}MRF4^{-/-}$ mice (4, 17), it is possible that the Shh signalling pathway requires one or both MRFs to regulate MyoD expression *in vivo*. A previous ChIP-on-chip study in limb buds did not identify MyoD as a direct target of the Gli3 protein (102). Thus, MyoD may be a direct target of Gli2, and not Gli3, during skeletal myogenesis. Our results are supported by previous reports, identifying Gli2 as a direct

regulator of other bHLH factors like Ascl1 (79) and Hes1 (103), suggesting the regulation of bHLH factors by Gli2 as a common function of Hh signalling during development.

Identification of molecular mechanisms regulating MyoD expression is important for the development of future transplantation studies, as stem cells lacking MyoD expression show better engraftment into muscle (8, 10, 11). The function of genomic regions identified in the Gli2-ChIP assay is currently not known. Notably, the region containing the *MyoD*A site, which was associated with Gli2 in this study (Fig. 4.3C), was also bound by MyoD and MyoG in a genome-wide ChIP-sequencing study (Table 4.2) and is well conserved in higher vertebrates (Fig. 4.3B, Appendix I Fig. S3). Since the deletion and/or mutation of known *MyoD* gene regulatory elements does not result in a complete loss of MyoD expression (104-106), there is at least partial redundancy between these elements and/or the existence of other putative novel regulatory elements.

The identification of an optimal cocktail of reagents leading to increased proliferation, but decreased activation and differentiation of SCs, would be beneficial for the successful expansion of SCs and their subsequent use for muscle engraftment and repair. The administration of cyclopamine *in vivo* decreases the number of activated $MyoD^+$ SCs following muscle injury (33) and increases the number of $Pax7^+$ muscle progenitor cells in developing zebrafish (107). Our results support and extend these reports, by showing for the first time that the inhibition of endogenous Hh signalling (Fig. 4.4) results in decreased levels of MyoD transcripts during SC activation *in vitro* (Fig. 4.5). Since *MEF2C* transcript levels are modulated by Hh signalling (Figs. 4.5 & 4.8), in agreement with previous reports (34, 57, 63), it is possible that Hh signalling may regulate MyoD expression at least in part via MEF2 (100, 108, 109). It is unlikely that Hh signalling regulates MyoD expression via

Pax7 (110), since inhibition of Hh signalling did not significantly affect Pax7 expression during SC activation *in vitro* (Fig. 4.5). In contrast to our findings showing no effect of Hh inhibition on *Myf5* expression (Fig. 4.5), Straface et al. reported fewer $Myf5^{+}$ SCs following cyclopamine administration in injured muscle (33). The difference in the results might be due to the application of cyclopamine prior to muscle injury (33), in comparison to the addition of cyclopamine after SC isolation (Fig. 4.5) or to different roles for Myf5 *in vitro* versus *in vivo* (111). Thus, similar to MAPK (92) and MLCK (36), inhibition of Hh signalling prevents upregulation of MyoD expression, indicating reduced SC activation.

MyoD protein activates the myogenic transcriptional machinery (112), regulating the formation, proliferation, and differentiation of myoblasts (21, 47, 113). We show that endogenous Hh signalling is activated by MyoD in myogenic conversion assays (Fig. 4.6), similar to the ability of MEF2 to regulate Hh signalling in *Drosophila* (114), and is important for MyoD function both on endogenous and exogenous promoters (Fig. 4.6 & Appendix I Fig. S5). Overexpression of Gli2 does not enhance myogenic conversion and slightly reduces upregulation of *MyoG* (Appendix I Fig. S5). In a previous study, overexpressed Gli1/2 inhibited MyoD protein function in reporter assays in C3H10T1/2 cells (97). It is possible that high levels of overexpression can cause squelching, or sequestering, of factors important for MyoD function, similar to TWIST proteins (115), or that different levels of Hh signalling can have different biological outcomes (116), such as inducing osteoblast differentiation in fibroblasts (117, 118) and generating different subtypes of ventral neurons in developing neural tube (119). KAAD-cyclopamine inhibits MyoD activation of endogenous gene expression to a greater extent than exogenous promoter activation (Fig. 4.6), possibly due to the loss of endogenous chromatin structure in transiently expressed plasmids (120). We also

show that Gli2, MEF2C and MyoD form a protein complex during C3H10T1/2 myogenic conversion and in differentiating P19 cells, which is capable of enhancing MyoD transcriptional activity on a skeletal muscle-related promoter (Fig. 4.7). Therefore, we demonstrate that MyoD activity is regulated by a complex of proteins, including MEF2C and Gli2, linking Hh signalling to MyoD function during muscle development.

Our results are analogous to findings in other systems, in which inhibition of Gli1-3 expression blocked ectopic neurogenesis induced by neurogenic bHLH factors (121). This is supported by the identification of gene ontology categories enriched in muscle cell differentiation and nervous system development (Table 4.3) from 519 genes containing conserved Gli, MEF2 and MyoD DNA binding clusters (Supplemental Excel File S2). The genes from the ion transmembrane transporter activity category (Table 4.3) may also represent cardiac muscle specific ion channels and might be co-regulated with the cardiogenic bHLH factor Hand1 (122). Thus, since Gli and MEF2 factors are expressed in developing heart (123, 124), brain (125-127) and skeletal muscle (6, 124), their ability to interact and modulate the activity of tissue-restricted bHLH factors like MyoD may represent a paradigm for the regulation of several cellular differentiation processes.

**Table 4.3. Selected gene ontology biological processes significantly enriched among genes containing Gli and MEF2 and MyoD conserved DNA binding clusters in UTR, promoter, intron and coding sequence regions.**

Altogether, 519 potential target genes were identified as described in (53). The background set of genes used was the entire mouse genome. For complete list of genes see Supplemental Excel File S2.

| Category | Targets | Fisher Exact P-value | Example genes |
|---|---|---|---|
| Regulation of Gene Expression | 127 | 2.3E-9 | Meis2, Ash2L, Ell2, Foxd4, Pax2, Tcf4, Tcf12, Spfq |
| Organ Development | 90 | 9.9E-9 | MEF2D, Tbx15, Tbx4, Ldb1, Igf1r, Runx2 |
| Cell differentiation | 85 | 4.3E-8 | Dhh, Rorb, Lama1, SRF, Pbx3, JunB |
| Nervous System Development* | 57 | 1.3E-8 | Lhx6, Hes1, Runx1, Sim1, Hoxb3, Nog, Kif7 |
| Ion Transmembrane Transporter Activity | 30 | 1.1E-2 | Abca1, Atp8a2, Cacna1g, Cacna2d1, Hvcn1, Kcnd2, Scn3a |
| Chromatin Organization | 21 | 1.0E-3 | Myst4, Smyd3, Smarcc2, Hdac5, Mll1, Smarca5 |
| Muscle Organ Development | 15 | 6.8E-4 | Smad7, Dmd, Foxp2, Rara, Met |
| Muscle Cell Differentiation | 10 | 7.4E-3 | MEF2C, Ntf3, Ttn, Wnt4 |
| Limb Development | 8 | 8.6E-2 | Lmx1b, Pbx1, Pcsk5, Notch2 |

*Neurogenic bHLH factors like NeuroD2 are known to bind some MyoD DNA response elements (128).

## 4.7 Conclusions

Although the ability of Hh signalling to modulate the expression of MyoD in the embryo and regenerating adult muscle is known, we hereby provide novel added mechanistic insight into this process and demonstrate the importance of endogenous Hh signalling in the regulation of MyoD expression during adult mouse SC activation *in vitro*. We propose a model, where Gli2, MEF2C and MyoD participate in a regulatory loop by inducing and maintaining each other's expression as well as forming a protein complex capable of inducing muscle-specific gene expression (Fig. 4.8B), linking Hh signalling to MyoD function and expression.

## 4.8 Authors' contributions

A.V. carried out P19 differentiation, C3H10T1/2 conversion assays, SC *in vitro* expansion, IF, QPCR analysis, ChIP assay with Gli2 antibodies, IP from C3H10T1/2 and P19[MEF2C-TAP] cells, reporter assays in C3H10T1/2 cells in the presence or absence of KAAD-cyclopamine and manuscript writing. E.C. analyzed ChIP assay with Gli2 antibodies, participated in IF and performed reporter assays with *MyoD* -2.2kb prom and Gli2 expressing plasmid, A.M. participated in SC *in vitro* expansion in the presence of SB and/or cyclopamine, J.F. participated in ChiP assay with Gli2 anitbodies, N.W.B., C.L. and G.L. extracted SCs from mice, V.W. and S.T. maintained and supplied $Ptch1^{+/+}$ and $Ptch1^{+/LacZ}$ mice, β-gal and Shh specific reagents, I.S.S. conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript. The authors indicate no potential conflicts of interest.

**4.9 Acknowledgements**

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# CHAPTER 5

# DISCUSSION

## 5.1 Hh signalling regulates unique steps of three P19 cell differentiation pathways

We show here that Gli2 mediates neurogenesis in P19 EC cells at least in part by directly regulating the expression of Ascl1/Mash1, a neurogenic bHLH transcription factor. We also demonstrate that during cardiac myogenesis in P19 EC cells, Gli2 and MEF2C associate with each other's gene elements and regulate each other's expression. Moreover, Gli2 and MEF2C form a protein complex, which participates in the regulation of cardiac muscle specific gene expression. Finally, we show that Hh signalling regulates skeletal myogenesis in stem cells by regulating the expression of MyoD and MEF2C and by participating in a protein complex consisting of Gli2, MEF2C and MyoD proteins, which enhances MyoD protein function to induce skeletal muscle gene expression. Thus, we propose a model, where Hh signalling via Gli2 regulates neurogenesis, cardiomyogenesis and skeletal myogenesis at unique steps of each pathway (Fig. 5.1).

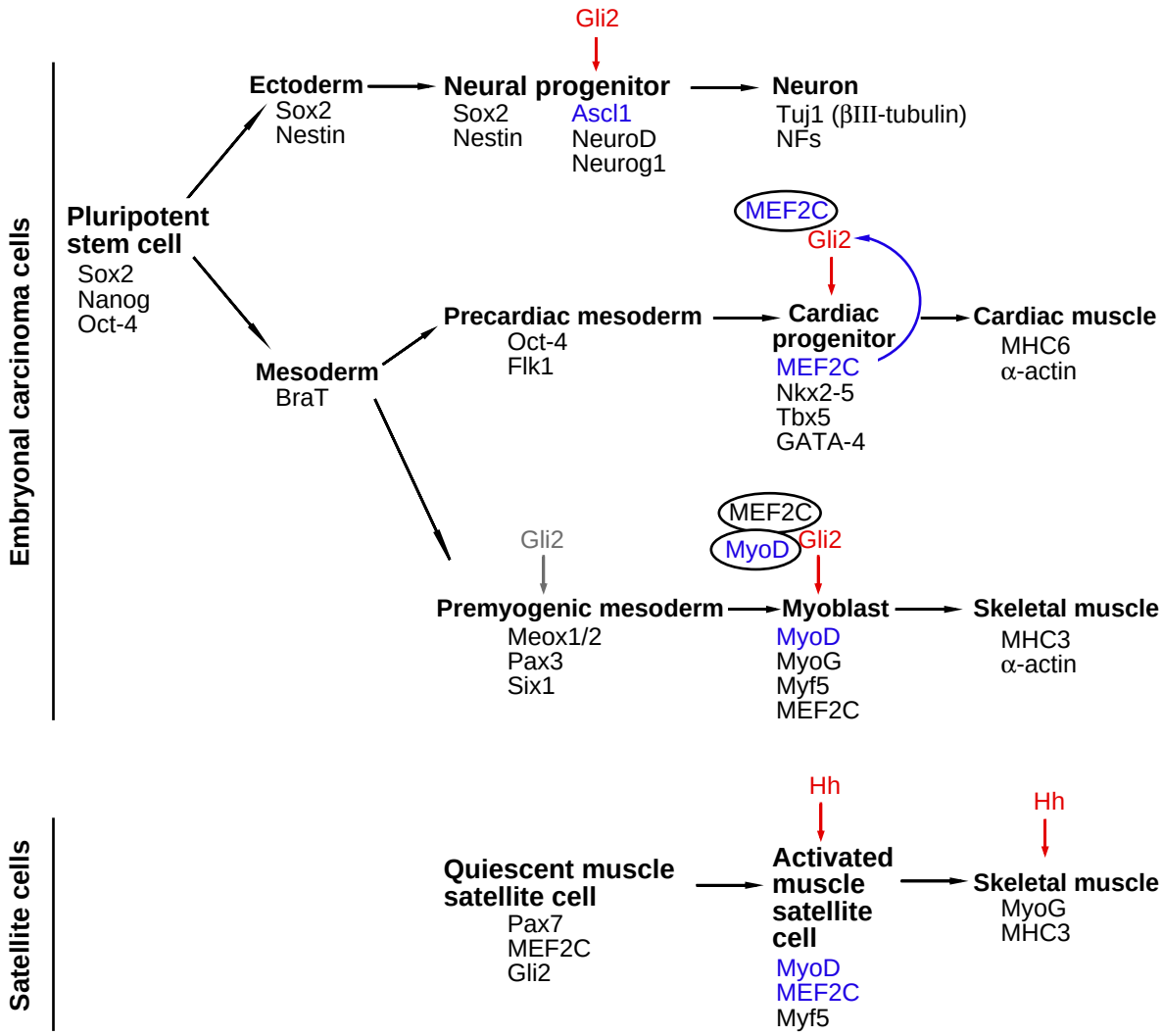
The results from Chapter 2 show that Gli2 induces neurogenesis in P19 cells at least in part by directly regulating Mash1 gene expression at the stage of neural progenitor cell formation. The identification of Mash1 as a novel target of Gli2 during P19 cellular neurogenesis supports and extends previous reports showing the regulation of neurogenic bHLH factors by Gli proteins *in vivo* (1-3) and in ES cells (4). During embryogenesis, the Mash1 transcription factor and notochord-derived Shh regulate neurogenesis in the developing CNS (reviewed in (5, 6)). In postnatal and adult brain, Shh is expressed in

cerebrospinal fluid and regulates the establishment and maintenance of SVZ and SGZ (subventricular and subgranular zone) brain stem cells (reviewed in (7-9)), which express Mash1 protein (10). Thus, our results may provide novel mechanistic insight into Shh-mediated neurogenesis *in vivo*.

In Chapter 3, we were interested to test if Gli2 and MEF2C co-regulated cardiomyogenesis in stem cells. We show that Gli2 and MEF2C enhance cardiomyogenesis at least in part by inducing each other's expression and binding each other's gene elements at the stage of cardiac muscle progenitor stage formation. Moreover, Gli2 and MEF2C form a protein complex capable of synergistically activating cardiac muscle gene expression. Our results identifying a novel mechanism for co-regulation of cardiomyogenesis by Gli2 and MEF2C are supported by the similar heart phenotypes in mice lacking Gli or MEF2C (11-14). During heart formation *in vivo*, Shh and Ihh are important for FHF development, as Shh/Ihh DKO and Smo KO mice show delayed Nkx2-5 expression in the heart tube and delayed heart formation (15). Since Shh is expressed in endoderm (16, 17) and in various midline structures (18), it is difficult to conclude which tissue is responsible for the cardiogenic effects of Shh ligand on FHF. Tissue-specific removal of Shh in endoderm results in defective outflow tract (OFT) (19), thus establishing a role for endodermal Shh during SHF development. MEF2C also regulates the differentiation of FHF and SHF during heart development (reviewed in (20)). Therefore, our results provide a novel mechanism of Gli2 and MEF2C mediated cardiomyogenesis in stem cells, which may be applicable to Hh and MEF2 orchestrated heart formation *in vivo*.

**Figure 5.1. Regulation of cardiomyogenesis, skeletal myogenesis and neurogenesis in stem cells at unique steps of the pathway.**

During neurogenesis in P19 cells, we show that Gli2 (in red) regulates the expression of neurogenic bHLH factors at the stage of neural protenitors (Chapter 2). During cardiomyogenesis in P19 cells, we demonstrate that Gli2 and MEF2C regulate each other's expression and cardiomyogenesis at the stage of cardiac progenitor cells, and that Gli2 and MEF2C form a synergistic protein complex (Chapter 3). During skeletal myogenesis in P19 cells, we show that Gli2 regulates the expression of MEF2C and MRFs at the stage of committed skeletal myoblasts and forms a protein complex with MyoD and MEF2C proteins, which is capable of enhancing MyoD transcriptional activity (Chapter 4). In addition, Gli2 is known to regulate the formation of pre-myogenic mesodermal cells and the expression of Pax proteins (21) (grey arrow). In mouse satellite cells, Hh signalling regulates the expression of MEF2C and MyoD in activated satellite cells and the expression of MyoG and MHC3 in differentiated cells (Chapter 4). Transcription factors that were found to be directly regulated by Gli2 are shown in blue. Proteins are shown in ovals.


Embryonal carcinoma cells
Pluripotent stem cell
Sox2
Nanog
Oct-4
Ectoderm
Sox2
Nestin
Gli2
Neural progenitor
Sox2
Nestin
Ascl1
NeuroD
Neurog1
Neuron
Tuj1 (βIII-tubulin)
NFs
Mesoderm
BraT
Precardiac mesoderm
Oct-4
Flk1
MEF2C
Gli2
Cardiac progenitor
MEF2C
Nkx2-5
Tbx5
GATA-4
Cardiac muscle
MHC6
α-actin
Gli2
Premyogenic mesoderm
Meox1/2
Pax3
Six1
MEF2C
MyoD
Gli2
Myoblast
MyoD
MyoG
Myf5
MEF2C
Skeletal muscle
MHC3
α-actin
Satellite cells
Quiescent muscle satellite cell
Pax7
MEF2C
Gli2
Hh
Activated muscle satellite cell
MyoD
MEF2C
Myf5
Hh
Skeletal muscle
MyoG
MHC3

Results from Chapter 4 demonstrate that Gli2 enhances skeletal myogenesis in P19 cells at least in part by directly regulating the expression of MyoD at the stage of committed skeletal myoblast formation. These results support and expand previous reports showing the regulation of myogenic bHLH gene expression by notochord-derived Shh during embryonic skeletal muscle formation (22-24). Since Hh signalling is known to regulate embryonic muscle formation (25-27) and adult muscle regeneration (28), we hereby provide a novel mechanism that may contribute to understanding regulation of muscle formation and regeneration by the Hh signalling pathway. We also show that endogenous Hh signalling is important for MyoD transcriptional activity in myogenic conversion assays and that Gli2, MEF2C and MyoD form a protein complex, which enhances MyoD activity on skeletal muscle related promoters. It is important to understand the regulation of MyoD protein function since mice expressing MyoD protein deficient in transcriptional activity exhibit impaired postnatal muscle regeneration (29). Thus, our results demonstrating a role of Hh signalling in the regulation of MyoD transcriptional activity may represent an important aspect of Hh-regulated muscle repair *in vivo*.

Our results illustrate that Gli2 directly regulates tissue-restricted factors like Mash1, MEF2C and MyoD. Apart from identification of Mash1 and MyoD, which are bHLH factors that we identified as novel direct targets of Gli2 (Chapters 2 and 4), other bHLH factors, such as Hes1 and Myf5, are known to be directly regulated by Gli2 from previous reports (30, 31). We also demonstrate that Gli2 interacts with MEF2C and MyoD proteins and that endogenous Hh signalling is important for MyoD transcriptional activity. Interestingly, Gli2 expression is known to be important for the transcriptional activity of neurogenic bHLH factors (2, 3). Thus, the ability of Hh signalling to regulate the expression and function of

bHLH proteins may indicate a novel paradigm in Gli-mediated regulation of differentiation programs, which may be applicable to other developmental programs in the embryo.

During embryogenesis, Hh ligands are broadly expressed in the embryo. Dhh is expressed in the gonads, Ihh is expressed in primitive endoderm and growth plates of the bones and Shh is expressed in midline tissues such as Hensen's node, notochord and floor plate, the zone of polarizing activity of the limb and pharyngeal endoderm (17, 18). As a result, Hh signalling regulates the development of various tissues in paracrine and endocrine fashion (18). While it is obvious that ES and EC differentiation does not fully recapitulate embryonic organogenesis, cellular aggregation to form 3D structures (EBs) follows early embryogenesis *in vivo* as it induces the formation of three germ layers and the formation and elongation of anterior-posterior axis (32). In addition, EBs have spatio-temporal expression of Hh ligands and Gli transcription factors, which resembles paracrine signalling, and respond to Hh signalling modulators (33, 34). Thus, our results demonstrating new mechanisms of Gli2-mediated neurogenesis, cardiomyogenesis and skeletal myogenesis in P19 cells should be applicable to the early events of cellular differentiation occurring during neural tube, heart and skeletal muscle formation. This is in agreement with previous reports, which demonstrate the role for Hh ligands during early events of neural tube patterning (6), heart formation (15) and somitogenesis (24, 35).

**5.2 Future directions**

ES cells represent a unique resource to generate specific cell types in the hopes of improving the function of failing organs, such as injured spinal cord (36), infarcted heart (37) or regeneration-deficient muscle in patients with muscular dystrophy disorder (38). Differentiation of ES cells using EBs is a beneficial means to dissect molecular mechanisms

regulating various differentiation programs. Unfortunately, the major pitfall with using EBs for generation of specific cell types for stem cell therapies lies in the fact that mesoderm, endoderm or ectoderm constitutes only a third of the germ layers. Moreover, each germ layer equally contributes to various arising cell types or derivatives. Thus, the differentiation efficiency of a particular cell type is usually very low (for example, skeletal muscle differentiation in ES cells is under 2%). Therefore, there is a need to enrich for specific cell types during ES differentiation. Moreover, according to the FDA regulations for the development of potential hES based stem cell therapies (39), there is a need to identify and adopt culture and differentiation conditions for hES cells that would, among other requirements, be non-human serum- and feeder-free. To develop these conditions, researchers can use the knowledge obtained for specific tissue formation during embryogenesis.

Neuronal cell differentiation can be relatively easily enriched in ES and EC cells treated with RA (40, 41) and can occur in the absence of serum with or without additional external factors (42, 43). Cardiomyogenesis can be induced in serum-free conditions using application of several exogenous ligands, including activin A and BMP-4 (44-46). Until now, there are no reports of serum-free directed skeletal muscle differentiation in ES cells, however, considerable progress has been made. Namely, low doses of RA or forced expression of Pax3/7 transcription factors enhance skeletal myogenesis in P19 EC as well as mouse and human ES cells (47-51). Our results showing the role of Hh signalling at specific steps of various differentation programs (Fig. 5.1) can potentially be used to develop or refine serum-free protocols for programs like neurogenesis, cardiomyogenesis and skeletal

myogenesis. In agreement, application of Shh and RA to differentiating ES cells results in better yields of motoneurons as compared to RA alone (52, 53).

While the development of serum- and feeder-free ES culture conditions are necessary to comply with FDA regulations, it is also important to know if derived cell types are functional. In the presence of Shh and RA, ES cells differentiate into functional motoneurons, which connect with appropriate muscle groups when transplanted into chick spinal cord (52, 53). RA-free differentiation protocols, on the other hand, generate dorsal telencephalic (forebrain) progenitor cells, which can be ventralized with application of Shh to yield functional GABAergic neurons (54). The differentiation of hES cells into cardiac muscle usually generates immature cardiomyocytes (reviewed in (37)). Nevertheless, hES-derived cardiomyocytes are known to engraft in infarcted hearts of animals and to contribute to remuscularization and the overall improvement of heart function (44, 55), however, it is not clear if these effects are sustained (56). In order for the effects to be sustainable, transplanted cells need to organize into a specific mature myocardium (e.g. ventricular and atrial) and be able to bear the workload of a recipient heart (reviewed in (37)). Thus, there is a need to study the regulation of cardiomyocyte maturation in ES cells. For skeletal muscle repair, there is at least one report showing successful engraftment of Pax3/7-induced hES derived muscle progenitor cells into acutely injured tibialis anterior (TA) muscle of immunodeficient mice and subsequent muscle recovery, however, the ability to repair dystrophic muscle by hES cell derivatives remains to be addressed (57). Moreover, the ability of hES-derived muscle progenitor cells to generate different functional muscle cells with properties of epaxial, hypaxial or limb musculature, and/or specific muscle fibre types is currently not known. This might be important for the development of muscle progenitor

therapies for patients with Duchenne muscular dystrophy or muscle wasting due to aging, where usually all skeletal muscle tissues are affected.

Another aspect that researchers have to tackle before stem cell therapies can be devised for human patients is a choice of a progenitor or more differentiated cell to repair damaged tissue. Studies have shown that adult mature cardiomyocytes have lower survival rates after transplantation as compared to fetal or neonatal cardiomyocytes (58). For spinal cord injury repair, neural progenitor cells from EBs or neural spheres derived via EBs that can generate neurons, glia and astrocytes, are known to be effective in engraftment in mice with spinal cord injury and subsequent functional recovery (reviewed in (36)). For skeletal muscle repair, muscle progenitor cells that do not express MRFs are known to better populate the satellite cell niche, as compared to $MRF^{+}$ myoblasts ((59-61) and reviewed in (62)). Thus, there is a need to develop a protocol for the generation of Pax3/7-positive muscle progenitors that do not yet express MRFs. At least two protocols are available to generate $Pax3/7^{+}$ $MRF^{-}$ progenitor cells from hES cells using application of exogenous RA (48) or expression of Pax3/7 proteins (51). Satellite cells can also be used to repair damaged skeletal muscle, however, SCs irreversibly upregulate the expression of MRFs when cultured *in vitro*, resulting in SC activation and differentiation (reviewed in (62)). Our results indicate that inhibition of endogenous Hh signalling downregulates MyoD and MyoG expression during SC activation *in vitro*, which is suggestive of reduced SC activation and differentiation (Chapter 4). Notably, different biological materials are also known to reduce SC activation when cultured *in vitro* (63). Thus, identification of a cocktail of reagents in combination with the use of biomaterials resulting in reduced SC activation and

differentiation would undoubtedly be beneficial for the development of adult stem cell therapies for muscle regeneration.

Recently, the first proof-of-principle study showed efficient delivery of hES cell derived retinal pigment epithelial cells to the eyes of human patients with macular degeneration. This demonstrated some functional visual improvement, and, importantly, did not result in teratomas, abnormal growth, inflammation or retinal detachment (64). Notably, the human eye, along with the brain, is immunologically privileged as it is normally protected by the blood-brain barrier from immune cells present in the rest of the body (65). This fact makes hES cell transplants in the eye resistant to immune rejection. This problem, as it applies to potential stem cell therapies for non-immunologically privileged organs, could have been addressed in the clinical trial of hES cell derived oligodendrocyte precursor mediated repair of spinal cord injury conducted by Geron. However, the trial was terminated prior to its completion (66). Nevertheless, although the report from Schwartz et al. and colleagues, demonstrating first efficacy and efficiency of hES cell therapy is preliminary and follow-up studies have to be performed (64), these findings will probably pave the way to other clinical trials using hES cells.

### 5.3 Limitations

While the results of this Ph.D. thesis demonstrate the role for Gli2 transcription factor during neurogenesis and cardiac and skeletal myogenesis in P19 EC cells (Chapters 2-4), their validation in mouse and human ES cells remains to be performed. To this end, we have started preliminary experiments and demonstrated that when the Hh signalling pathway is activated, mouse and human ES cells exhibit more efficient expression of cardiac muscle progenitor cell specific transcription factors (Fair, Voronova and Skerjanc, unpublished

observations). It is likely that the results demonstrating a role for Hh signalling in P19 EC cells will be reproduced in mouse and human ES cells, similarly to RA signalling (47, 48).

It was previously demonstrated that cyclopamine inhibited Hh signalling via specific binding to Smo receptor (67) and the results obtained with cyclopamine in a variety of tumour cells mimicked the results obtained with a specific knock down of Smo mRNA expression (68-70). Moreover, the effects of cyclopamine can be rescued with forced expression of Gli1, a positive regulator of the Hh pathway acting downstream of Smo (70). Interestingly, Meyers-Needham et al. has recently shown that cyclopamine can activate nNOS pathway to induce apoptosis independently of Smo/Gli inhibition (71). Importantly, we have not observed cytotoxic effects of cyclopamine or Hh-specific antibodies on satellite or C2C12 cells (Voronova, Skerjanc, unpublished observations). Thus, although the results obtained with cyclopamine in Chapter 4 are likely to be specific to the inhibition of the Hh signalling pathway, the role of Hh-Gli signalling in satellite and C2C12 cells in Chapter 4 should be verified using other approaches, such as siRNA technique. The knock down of Gli2 using siRNA approach in P19 cells could also complement the use of Gli2 dominant negative chimeric protein in Chapters 2-4.

Lastly, although satellite cells treated with Hh signalling inhibitors exhibited reduced MyoD and MHC3 expression (Chapter 4), which is indicative of reduced activation and differentiation, the function of these cells remains to be tested. We have initiated preliminary experiments showing that the effect of cyclopamine on satellite cells can be successfully reversed (Voronova, Skerjanc, unpublished observations), thus making the Hh signalling pathway an attractive subject to study during the regeneration of muscle by satellite cells.

## 5.4 Conclusions

The results obtained in this project provide a link between the regulation of tissue-restricted factors like Mash1, MEF2C and MyoD, and a general signal-regulated Gli2 transcription factor. Thus, we provide novel mechanistic insights into the neurogenic, cardiogenic and myogenic properties of Gli2 *in vitro*, and offer novel plausible explanations for its *in vivo* functions.

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# Appendix G Q-PCR primer sequences

| Target | Forward primer | Reverse Primer |
|---|---|---|
| Ascl1 | ACTTGAACTCTATGGCGGGTT | CCAGTTGGTAAAGTCCAGCAG |
| β-actin | AAATCGTGCGTGACATCAAA | AAGGAAGGCTGGAAAAGAGC |
| BraT | CTGGACTTCGTGACGGCTG | TGACTTTGCTGAAAGACACAGC |
| CyclophilinB | GATGGCACAGGAGGAAAGAG | AACTTTGCCGAAAACCACAT |
| Dhh | AGCCATCGCGGTGATGAAC | CAGTCACACGTAGGCGTACTC |
| GATA-4 | AAAACGGAAGCCCAAGAACCT | TGCTAGTGGCATTGCTGGAGT |
| GFAP | CCAAGCCAAACACGAAGCTAA | CATTTGCCGCTCTAGGGACTC |
| Gli/EnR | GGAGAGTGTGGAGGCCAGTA | CTGGGTTCCGGCTGTCTCT |
| Gli1 | CCAAGCCAACTTTATGTCAGGG | AGCCCGCTTCTTTGTTAATTTGA |
| Gli2 | CAACGCCTACTCTCCCAGAC | GAGCCTTGATGTACTGTACCAC |
| Gli3 | AGCAACCAGGAGCCTGAAGTC | GTCTTGAGTAGGCTTTTGTGC |
| Ihh | GACGAGGAGAACACGGGTG | GCGGCCCTCATAGTGTAAAGA |
| MEF2C | TCTGTCTGGCTTCAACACTG | TGGTGGTACGGTCTCTAGGA |
| MEF2C-TAP | TGGCAACAGCAACACCTACATAA | GGGGTGGTGGTACGGTCTCTA |
| MHC3 | GCATAGCTGCACCTTTCCTC | GGCCATGTCCTCAATCTTGT |
| MHC6 | CAACAACCCATACGACTACGC | ACATCAAAGGGCCACTATCAGTG |
| Myf5 | CCTGTCTGGTCCCGAAAGAAC | GACGTGATCCGATCCACAATG |
| MyoD | CCCCGGCGGCAGAATGGCTACG | GGTCTGGGTTCCCTGTTCTGTGT |
| MyoG | GCAATGCACTGGAGTTCG | ACGATGGACGTAAGGGAGTG |
| Nanog | TCTTCCTGGTCCCCACAGTTT | GCAAGAATAGTTCTCGGGATGAA |
| Nestin | CCCTGAAGTCGAGGAGCTG | CTGCTGCACCTCTAAGCGA |
| NeuroD | GCATGCACGGGCTGAACGC | GGGATGCACCGGGAAGGAAG |
| Neurog1 | CCAGCGACACTGAGTCCTG | CGGGCCATAGGTGAAGTCTT |
| Nkx2-5 | AAGGAACAGCGGTACCTGTC | GCTGTCGCTTGCACTTGTAG |
| Oct-4 | CAGCCAGACCACCATCTGTC | GTCTCCGATTTGCATATCTCCTG |
| Pax3 | TTTCACCTCAGGTAATGGGACT | GAACGTCCAAGGCTTACTTTGT |
| Pax7 | CTCAGTGAGTTCGATTAGCCG | AGACGGTTCCCTTTGTCGC |
| Ptch1 | AAAGAACTGCGGCAAGTTTTTG | CTTCTCCTATCTTCTGACGGGT |
| Shh | AAAGCTGACCCCTTTAGCCTA | TGAGTTCCTTAAATCGTTCGGAG |
| Smo | CTTGGTGCGAACAGACAACC | GGTAGCGATTGGAGTTCCGC |
| Sox2 | GACAGCTACGCGCACATGA | GGTGCATCGGTTGCATCTG |
| Tbx5 | CTTTCGGGGCAGTGATGAC | TTGGATGAGGTGGAGAGAGC |

# Appendix H ChIP-QPCR primers

| Target gene | Forward primer | Reverse Primer |
|---|---|---|
| Ascl1 A | CTGGACTCACTGGGTGGTCT | AGAGGCTGCTAGCCATGTGT |
| Ascl1 B | TCTTTCTCTGTCGCCATTCA | GGACGCTCCGGTTTGTATAG |
| Ascl1 C | TTCTTTGAGGCCTCTTCTTCA | TGAAATGCTGACCTCTTCCA |
| Ascl1 D | CCTAAGATCAATGGGCCAAA | CCCACCCAACTGTCCTAGAG |
| Gli1 | GCACCCCCTCTCTAGCTTCTATC | GGACCACCCGCGAGAAGCGCAAACT |

# Appendix I Supplementary Figures to Chapter 4

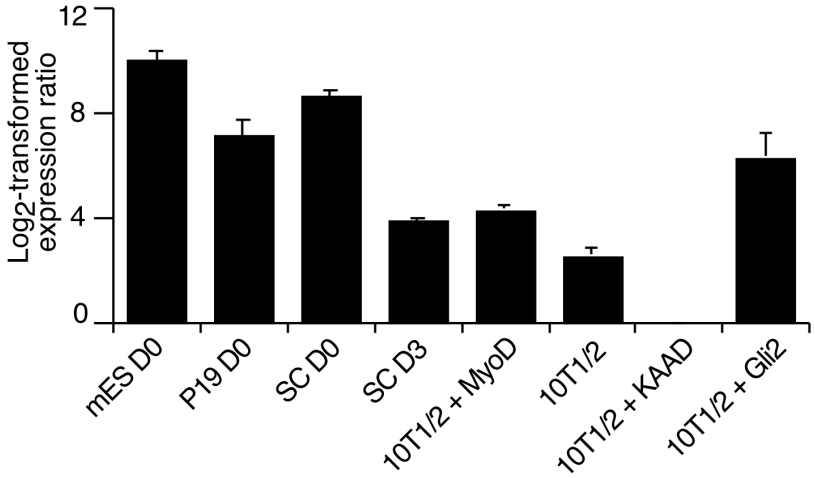


Fig. S1 QPCR analysis of Gli1 mRNA expression in mouse ES (mES), P19, satellite cells (SC) and 10T1/2 fibroblasts transfected with MyoD or Gli2 or treated with or without KAAD-cyclopamine (KAAD). Data is relative to 10T1/2 cells treated with KAAD-cyclopamine. Error bars represent SEM from three biological replicas (n=3). D=day.

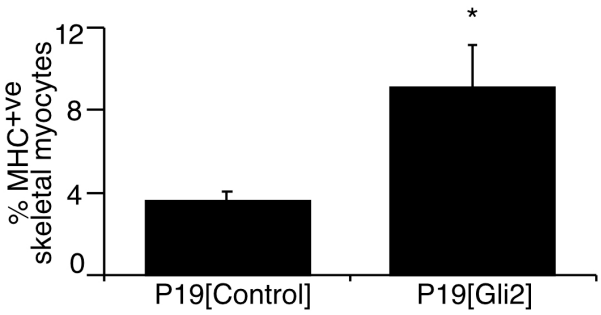


Fig. S2 MHC-positive skeletal myocytes from Fig. 2A were counted in 10 random fields and expressed as % of the total number of nuclei, n=4. In total, 20,000 cells were counted.*$p<0.05$

**Fig. S3**

The regions corresponding to the following coordinates were analyzed for conservation and the presence of conserved Gli (purple, corresponds to *MyoD* A site in Fig. 4.3A-C), MyoD (blue) and MyoG (green) binding sites using Mulan engine and Transfac elements as described in Materials and Methods:

mm9 chr7:53595007-53596449
hg18 chr11:17664117-17665569
rn4 chr1: 9687290-96874350
canFam2 chr21:43415158-43416625
equCab2 chr7: 85947287-85948752

```
canFam2_dna     TAGAGGGATCAGAGTTCTGTTGGAGGCTCTGCAATGAACAGGCTGTGTGGCCTCGGGAAG
equCab1_dna     TAGAGGGCTCAGAGTCGTAGTGCAGGCTCTGCTGCGAACAGACTGTGTGACTTTGGGTAG
hg18_dna        GAGAGAGACCGGGGCTCTG---------CTGTGAACAA----GCATGTGACCTTAGGCAG
rn4_dna         AAGG----CTAGAGTTCTTGAACAGTTTCTGCCATCA----------TGACCTTGGGCAG
mm9_dna         GAGGGAAGCCAGATCTCTGAAGCAATTCCTGTCATCA----------TAACCTCGGGCAG 81
                 **        *     *          ***     *          *  * *  ** **

canFam2_dna     GTCCCTCACCTCTCTGGGCCCCAGTTTCCTGTTCTTTAAGGTGAGGGGCTGAGACCAGAG
equCab1_dna     CTGCCTCACGTCTCTGGGCCCTAGTTCCAT-TTCTTTAAGATGAGGGACTTAGACAGGAG
hg18_dna        GTGCCTCACCAACCTAGGCTCCAGTTTTCTTTTCTTTAAGGTGAGGGGCTTAGACAGGAG
rn4_dna         GTGTCTC-CTGATCTGGGCTCTTGTTTCCTTCTCTTAAAGGTGAGGGGTTCAGATGGGAT
mm9_dna         GTGTCTC-CTGATCTAGGTTCTTGTTTCCTTCTCTTTACGGTGAGGGGTTCAGATGGGAT 140
                 *  *** *    ** **  *  ***   *  **** * * ******  * ***   **

canFam2_dna     GTGCTCTGAGAATCAGTGGTTGTGATCCTTAGGGGCTTAGGCAGAGGTCCCTGGATAGCT
equCab1_dna     ATGCTCTGAGAATTGGTGCATGTGATCCTTAGGGGCTTAGGCAGAGGACTCGGGACAGCC
hg18_dna        GAGCTCTGAGAGTCTGCGGATGTTATCCTTGCGGGCTTAAACAGAGGTCCCTGGACAGCC
rn4_dna         ATG--CTGGGGTTCTGCAGAAGCCAGCCCTGGGGGCTTGGGCAGAAGTCTCTAGACAGCA
mm9_dna         GTG--CTGGGGGTCTGTGAAAGCCACCTCTGAGGGCTTAGGCAGAGGTCTCTAGACAGCA 198
                  *  *** *  *  *     *  * *  *  ******   **** * * *  ** ***

canFam2_dna     GGGATTTT-CTGAACCCCGCCATGGCGTCCCTGGCCTGGCTAGGAGCTCCCGCAGGGCAG
equCab1_dna     GAGATTTT-CTGAGCCCCGCCATGGCGTCCCTGGCCTGGCTAGGAGCTCCCGCAGGGCAG
hg18_dna        AGGATGTT-CTGAGCCCCACCATGGCGTCCCTGGCCTGGCTAGGGGCTCCCGCAGGGCAG
rn4_dna         GGGATTTT-CTGAGCCTCGCCACGAAGTCCCTGGCTTAGCTAGGGGCTCCTGCAGGGCAG
mm9_dna         GGGATTTTTCTGAGCCTCGCCATCAAGTCCCTGGCTTGGCTAGAGGCTCCTGCAGGGCAG 258
                  *** ** **** ** * ***    ********* * *****  ***** *********

canFam2_dna     GATCACATTGCCCCAGATTCCAGGACTGGCTGCAGCACAGACCCAGGATCGGGTGACAGA
equCab1_dna     GATCACATTGCCCCAGATTCCAGGACTGGCTGAGGCACAGACCCAGGATCGGGTGACAGA
hg18_dna        GATCACGTTGCCCCAGATTCCAGGACTGGCTGCGGCACAGACCCAGGATCAGGTGACAGA
rn4_dna         GATCACATTGCCCCAGATTCCAAGACTGGCTGTAGCACAGACCTGGGATCGGGTGACAGA
mm9_dna         GATCACATTGCCCCAGATTCCAAGACTGGCTGTAGCACAGACCTGGGATCGGGTGACAGA 318
                ****** *************** *********  *********  ***** *********

canFam2_dna     TCCATCCAGCCTGGACCGGCTCAACCTCTCAGCTACAGTCGCAGCCATGGGGGCTGGAAA
equCab1_dna     TCCATCCAGCCTGGACTGGCTCAACCTCTCAGCTACAGCCGTAGCCATGGGGGCTGGAAA
hg18_dna        TCCATCCAGCCTGGACTGGCTCAATCCCTCCACTACAGCCGCAGCCCTGGGGGCTGGAAA
rn4_dna         TCCTTCCAGCCTGGACTGGCTCAACCCTTCTGCTACAACAGCAGCCATGGGGGCTGGAAA
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mm9_dna          TCCTTCCAGCCTGGACTGGCTCAACCCTTCTGCTACAGCAGCAGCCATGGGGGCTGGAAA 378
                 *** ************ ******* *  **  *****   * **** *************

canFam2_dna      ACCAGAGCTAATGCAAGCCAGCTGGAGGCTAGGACAAGATTGATGAAGGACAGGGAGAGG
equCab1_dna      ACCAGAGCTAATGCAAGCCAGCTGGAGGCCAGGACAAGATTGATGAAGGACAGGGAGAGG
hg18_dna         ACCAGAGCTAATGCAAGCCAGCTGGAGGCCAGGACAAGATTGATGAAGGATAGGGAGAGG
rn4_dna          ACCAAAGCTAATGCAAGCCAGCTGGAGGCCAGGACAAGATTGATGAGGGACAGGGAAAGG
mm9_dna          ACCAGAGCTAATGCAAGCCAGCTGGGGCCAGGACAAGATTGATGGGGGACAGGGAAAGG 438
                 **** ******************** *** ***************  *** ***** ***

canFam2_dna      ATTTGGCCAGGGGGC---------TGGGCTGG----GGGGT--------------------
equCab1_dna      ATTTGGCCAGGGGGT---------TG----GG----GGGCT--------------------
hg18_dna         ATTTGGCCAGGGTTTTTTTCAGTGCG----GG----AGGGT--------------------
rn4_dna          -TTTGGCCAAGGTGT------GCATG----GGGGGTGGGGTAGGGTGTGTGAAGGGAGC-
mm9_dna          -TTTGGCCAAGGTGT------GCATG----GA----GGGGT-GTGTGTGTGGAGGGAGCA 482
                  ******** **             *    *      ** *

canFam2_dna      ------------AGAGCATAATTAGTCCTGCCTGCTTCCCA--GGGGCCCCCAAGCTTAC
equCab1_dna      ------------AGAGCATAATTAGTCCTGCTTGCTTCCCA--GGGGCCCCCAAGCCGGG
hg18_dna         ---------GGTAGAGAATAATTAGTCCTGCCTGCTTCCCA--GAGGCCCTCAAGCTGGG
rn4_dna          --AAGGAAGGGGAGGGAATAATTAGTCTCGCCTGCTTCCCAGGGGGTTCCCCATGCCTTG
mm9_dna          GTAAGGAGGGGGAGGGAATAATTAGTCTTGCCTGCTTCCCA-GGCGTTCCCCATG----- 536
                             ** * **********  ** *********  * *  ** ** *

canFam2_dna      TAAACCTCATTAATTGGGAACCCCCAGTGGGGAATTCCTGCTCATGACCCAGAGAAGGAA
equCab1_dna      TAAACCTCATTAATTGGGAACCCCCAGAGGGGAGTTCCTTCTCATGACCCAGAGAAGGAA
hg18_dna         CAAACTTCATTAATTGGGAACCCCCAGTGGGGAATTCCTGCTCATGACCCAGAAAAGGAA
rn4_dna          TAAACCTCATTAATTGGGAACCCCCAGTGGGGAACGTCTGCTCATGACCCAGAGAAGGAA
mm9_dna          ----CCTCATTAATTGGGAACCCCCAGTGGGGAATGTCTGCTCATGACCCAGAGAAGGAA 592
                     * ********************* *****    ** ************* ******

canFam2_dna      GGGCCAGTTAGGAGTTAATTAGTAGTG-AAAGTGCCAATCGGGG--AGTGTTCAAACTAT
equCab1_dna      GGGCTAGTTAGGAGTTAATTAGTAGTG-AAAGTGCCAATCGGGGAAAGTGTTTAAACTAT
hg18_dna         GAGCTCTTTAGGAGTTAATTAGTAGTG-AAAGTGCCAATCAGGGAAAATGTTTAAACTAT
rn4_dna          GGGCTTGTTAGGAGTTAATTAGTAGTG-AAACTGCCAAT-GGGGAAAGTGTTTCAACTAT
mm9_dna          GGGCTTGTTAGGAGTTAATTAGTAGTGAAAACTGCCAATCGAGGAAGGCGTTTAAACTAT 652
                 * **   ******************** *** *******   **     ***  ******

canFam2_dna      TAAATGACTGAGCACCTGTTTACCTACAAACAACTGATATGTCAATTATCTCGCCATCAA
equCab1_dna      TAAATGGCTGAGCACCTGTTTACCTACAAACAAATGATATGTCAATTATCTGACCGTCAA
hg18_dna         TAAATGTCTGAGCACCTGTTTACCTACAAACAACTGATACAGTAATTATCTCACCATCAA
rn4_dna          TAAGTGGCTGAACACCTGTTTATCTACAAACAACTGATATATCAATTATCTGTTCACCAA
mm9_dna          TAAGTGGTTGAACACCTGTTTATCTACAAACAACTGATATATCCATTATCTGTTCATCAA 712
                 *** **  *** ********** ********** *****     *******   *  ***

canFam2_dna      AGCAGGTCCCCTAAGATAGCGATTACCAACCTCGGGTGATCCTCAAGGTCACCTAGGAAC
equCab1_dna      AGCAGGTCCCCTAAGGTAGTGATTACCAACCTTGGGTGATCCTCAAGGTCACCTAGGAAC
hg18_dna         AGCAGGTCCTCTATGATAGTGATTACCAACCTCAGGAGAGCCTGAGGATCGCCTAGGAAC
rn4_dna          AGCAGATCCCCAACAATAGTGATCC---AACTCAGT-GACTTTGAGGGTCATTCGAAAAC
mm9_dna          AGCAGATCGCCAATGATAGTGATTTGAAGACTCAGT-GATCTCGAGGGTCATTCGGGAGC 771
                 ***** **  * *   *** ***       **  *  **     * * **       * *

canFam2_dna      CTTGTTAAGCATCAGTTCCCAATGCAGTGATCCTGATTGATTGGTCAGGGGTGAGAACTG
equCab1_dna      CTTGTTAAGCATCGGTCTCCAATGCAGTGATCCTGATTGGTCAGCTA-GGGTGAGGACCA
hg18_dna         CTTGTTAAGCATCCATCTCCAATGCAATGATCCTGATTGGTCAG-CAGGAGTGAGAAGCA
rn4_dna          TGTGCTATCTCTCAGCTTCCAACACAGTGATCCTTCTTGGTCAGCCAAAGCTGACAGTTA
mm9_dna          TGTGCTTTCTATCAGTGTCCAACATAGTGATCCTGCTTGGTCAGCCAGGGGTGACAGCCA 831
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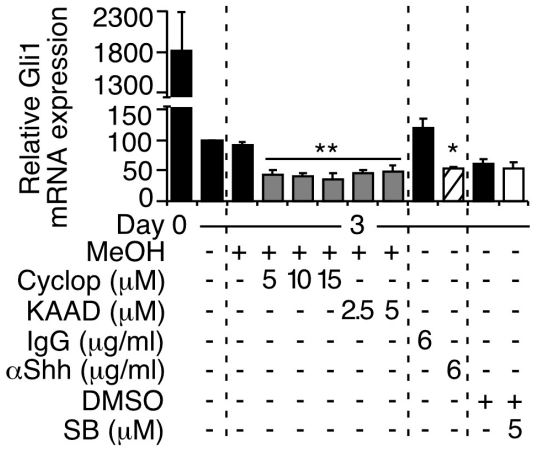


Fig. S4 QPCR analysis of Gli1 mRNA on day 0 (untreated) and day 3 of cultured mouse SCs in the presence or absence of cyclopamine (cyclop), KAAD-cyclopamine (KAAD), SB203580 (SB) and their respective vehicles (MeOH; methanol) or in the presence of Shh-specific or IgG non-specific antibodies for 3 days. Data from treatment with a specific reagent and its respective vehicle is separated by dashed line and expressed as a percentage of day 3 levels. Error bars represent +/- SEM (n=3). *p<0.05, **p<0.01.

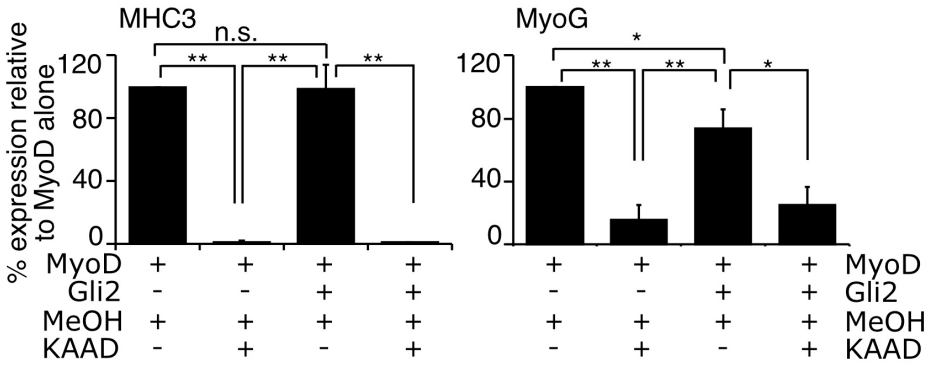


Fig. S5 Treatment of C3H10T1/2 cells with KAAD-cyclopamine reduces transcriptional activity of MyoD with or without Gli2. QPCR analysis of MHC3 and MyoG mRNA in C3H10T1/2 fibroblasts after transfection with MyoD and/or Gli2 expressing plasmids and subsequent 48h incubation in starvation media with KAAD-cyclopamine (KAAD) or vehicle (MeOH, methanol). Data was calculated as fold change over C3H10T1/2 fibroblasts transfected with an empty vector as a percentage of MyoD alone. Error bars represent +/- SEM from three biological replicas (n=3). *p<0.05, **p<0.01.

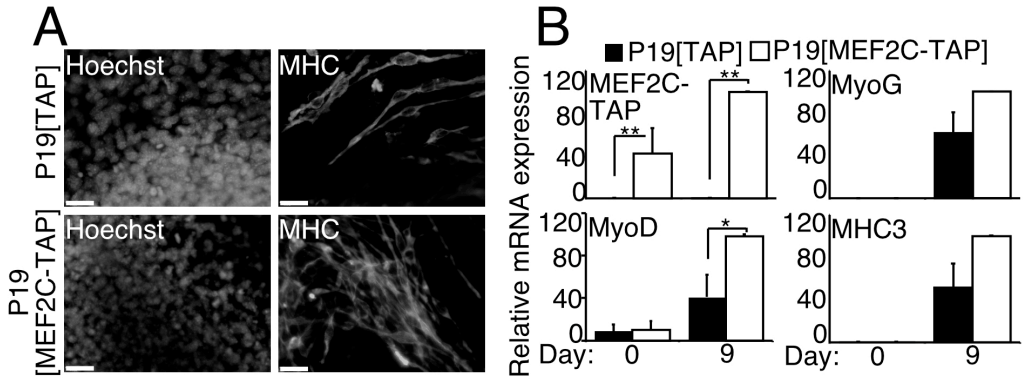


Fig. S6 (A): Day 9 differentiated P19[TAP] and P19[MEF2C-TAP] cells were reacted with MF20 antibodies to detect MHC expression. Nuclei were stained with Hoechst dye. Scale bar is 30 μm. (B): Overexpression of MEF2C upregulates MyoD and MyoG expression while enhancing skeletal myogenesis in P19 EC cells. QPCR analysis of the expression of indicated genes in P19[TAP] (black bars) and P19[MEF2C-TAP] (grey bars) cells at the time shown. Error bars represent +/- SEM from two clonal populations and two biological replicas (n=4), *p<0.05 and **p<0.01. n.s.=not significant.

# Appendix J Curriculum Vitae

**NAME: Anastassia VORONOVA**

**QUALIFICATIONS**

| Degree | Date | Institution |
|---|---|---|
| Doctor of Philosophy in Biochemistry | In progress | University of Ottawa, Canada |
| Master of Science in Gene Technology | 2007 | Tallinn University of Technology, Estonia |
| Bachelor of Science (Cum Laude) in Gene Technology | 2005 | Tallinn University of Technology, Estonia |

| Additional qualifications | Date | Institution |
|---|---|---|
| Foundations of Project Management I | 2012 | MITACS Step Program, University of Ottawa, Ottawa, Canada |
| Weinstein 2010 Satellite Hands-on Course on Morphology of Congenital Heart Disease | 2010 | Leiden University Medical Center, Leiden, Netherlands |
| BioXAS Practical course | 2005 | EMBL, Hamburg, Germany |
| Laboratory Animal Husbandry | 2003 | Tallinn University of Technology, Estonia |

**HONOURS AND AWARDS**

| Year | Description |
|---|---|
| Sep 2011 – Aug 2012 | Ontario Graduate Scholarship (OGS, Ontario Government, Canada) for 5$^{th}$ year of Ph.D. studies |
| 2011 | Poster award at the Ottawa Cardiovascular Symposium, Ottawa, Canada |
| 2011 | 2$^{nd}$ prize seminar award for Ph.D. students at the seminar day in the department of Biochemistry, Microbiology and Immunology, University of Ottawa |
| 2010 | Fisher Scientific Award of excellence in graduate studies, University of Ottawa, Canada |
| 2010 | Weinstein 2010 Travel Award at the Weinstein Cardiovascular Development Conference, Amsterdam, Netherlands |
| 2009 | Honorable mention award (equals to third prize) at CIHR National Poster |

| | |
|---|---|
| | Competition, Winnipeg, Canada |
| 2009 | First prize poster award at the OISB (Ottawa Institute of Systems Biology) Meeting "Progress in Systems Biology: the Brain and Mind", Ottawa, Canada |
| 2008 | Estonian Biochemical Society Award, Award for the first-author publication for M.Sc. level students in Estonia |
| Jul 2008 – Jun 2011 | Heart and Stroke Foundation of Canada Doctoral Research Award, Canada |
| Sep 2007 – Apr 2012 | University of Ottawa Excellence Scholarship, Scholarship to cover Ph.D. tuition fees for international student |
| 2007 | Estonian Biochemical Society Award, Award for the first-author publication for M.Sc. level students in Estonia |
| Feb 2007 – Mar 2007 | International Scholarship from SA Archimedes EU, Estonia, Support to obtain training in international laboratory |
| 2005 | Artur Lind Award, Estonia. Award for students in Estonia studying Gene Technology |
| 2005 | Cum Laude B.Sc. award, Tallinn University of Technology, Estonia |

**EXPERIENCE**:

**Positions held**:

| | |
|---|---|
| Sept 2007 - present | Ph.D. candidate in laboratory of Dr. Ilona Skerjanc, University of Ottawa, Canada |
| Jan – March 2007 | Laboratory of Dr. Illimar Altosaar, University of Ottawa, Canada, Visiting Scientist |
| Aug 2006 – Jan 2007 | FIBROTEX OÜ, Tallinn, Estonia, Technician specializing in proteomics |
| Jan 2005 – Sept 2007 | Tallinn University of Technology, Tallinn, Estonia, Research Assistant |
| Sept 2005 – Jun 2007 | M.Sc. student in laboratory of Dr. Peep Palumaa, Tallinn University of Technology, Estonia |
| Jan 2004 – Jun 2005 | B.Sc. student in laboratory of Dr. Peep Palumaa, Tallinn University of Technology, Estonia |

**Supervisory skills**:

- Erin Coyne – 4th year project "Regulation of MyoD expression by Gli2 in P19 EC cells", 2011, University of Ottawa, Canada
- Joel Fair – 4th year project "Characterization of stable Gli2-expressing mouse embryonic stem cells", 2010, University of Ottawa, Canada
- Joel Fair – summer project "Generation of stable Gli2-expressing mouse embryonic stem cells", 2010, University of Ottawa, Canada
- Anna Fischer – summer project "Determining direct targets of Gli2 during stem cell differentiation programs by ChIP analysis", 2009, University of Ottawa, Canada
- Flavia Sendi-Mukasa – Co-op project "Pax3 interactions in skeletal myogenesis of embryonal carcinoma cells", 2008, University of Ottawa, Canada
- Niina Sokolova – Master of Science thesis on "Effect of Zn(II) ions on redox equilibria of copper chaperone COX17", defended in 2008, at Tallinn University of Technology, Estonia
- Madis Sarapuu – summer project "Expression and purification of recombinant human Cox17 protein", 2006, Tallinn University of Technology, Estonia
- Niina Sokolova – Bachelor thesis on "New method for expression and purification of monomeric and dimeric recombinant human Cox17"; defended in 2006, at Tallinn University of Technology, Estonia
- Marina Tuuling – Bachelor thesis on "Expression and purification of recombinant Cox17 and its deletion mutant"; defended in 2005, at Tallinn University of Technology, Estonia

**Memberships**:

- Member of the Estonian Biochemical Society in good standing since 2004
- Member of Biochemistry Microbiology Immunology Graduate Student Association since 2008:
    - Active member-at-large from 2010 to present
    - VP Finance from 2009 to 2010
    - Active member-at-large from 2008 to 2009
- Active member of VROC (Virtual Researcher on Call) in good standing since 2008
- Member of International Student Workgroup (U. of Ottawa, GSAED) 2008-2009
- Member of International Society for Stem Cell Research since 2011.

**Organized conferences**:

"Research Can Lead You Anywhere" Career Day, January 2010, University of Ottawa, Ottawa, Canada

**Seminars as a guest speaker:**

"The role of Gli2 in stem cell lineage specification", 2009, Tallinn University of Technology, Tallinn, Estonia

**Outreach activities:**

2010 - "From stem cells to stem cell therapy" for "Reaching Every Student" program, Virtual Researcher on Call (VROC), between University of Ottawa and Peel District School Board, Toronto, Canada

2012 – "My Journey in Stem Cell Research" for "4U Bio Talks" program in Glebe Collegiate Institute, Ottawa, Canada

**PUBLISHED REFEREED PAPERS:**

1. **Voronova A.**, Al Madhoun A., Fischer A., Shelton M., Karamboulas C., and Skerjanc I.S. "Gli2 and MEF2C activate each other's expression and function synergistically during cardiomyogenesis in vitro" **Nucleic Acids Res.** (2012) 40: 3329-3347. **Featured article** (Featured Articles represent the **top 5%** of NAR papers in terms of originality, significance and scientific excellence)
2. **Voronova A.**, Fischer A., Ryan T., Al Madhoun A., and Skerjanc I.S. "Ascl1/Mash1 is a novel target of Gli2 during Gli2-induced neurogenesis in P19 EC cells" **PLoS ONE** (2011) 6, e19174
3. Avdic V., Zhang P., Lanouette S., **Voronova A.,** Skerjanc I., and Couture J. F., "Fine-tuning the stimulation of MLL1 methyltransferase activity by a histone H3 based peptide mimetic" **FASEB J.** (2011) 25, 960-967.
4. Gianakopoulos P. J., Mehta V., **Voronova A.**, Cao Y., Yao Z., Coutu J., Wang X., Waddington M. L., Tapscott S. J. and Skerjanc I.S. "MyoD directly upregulates premyogenic mesoderm factors during induction of skeletal myogenesis in stem cells" **JBC** (2011) 286, 2517-2525.
5. Savage J., **Voronova A.**, Mehta V., Sendi-Mukasa F. and Skerjanc S. I. "Canonical Wnt signaling regulates Foxc1/2 expression in P19 cells" **Differentiation** (2010) 79, 31-40.
6. **Voronova A**., Meyer-Klaucke W., Meyer T., Rompel A., Sillard R., and Palumaa P. "Oxidative switches on functioning of mammalian copper chaperone Cox17", **Biochem. J.** (2007) 408, 39-48.
7. **Voronova A**., Kazantseva J., Tuuling M., Sokolova N., Sillard R., Palumaa P., "Cox17, a copper chaperone for cytochrome c oxidase: Expression, purification, and formation of mixed disulphide adducts with thiol reagents." **Protein Expr. Purif.** (2007) 53, 138-144.
8. Palumaa P., Kangur L., **Voronova A**., Sillard R., "Metal-binding mechanism of Cox17, a copper chaperone for cytochrome c oxidase." **Biochem. J.** (2004) 382, 307-314.

**ACCEPTED REFEREED PAPERS:**

9. Wan S., Goto K., Staebler J. M., Mottiar Y., Johnson A. M., **Voronova A.**, Blais D. R., Zaidi M. A., and Altosaar I. "Bacterial nitrous oxide reductase expressed in transgenic plants: evidence for sufficient anaerobicity to permit activity" (2012) **Can. J. Plant. Sci.**, Accepted

**IN REVISION MANUSCRIPTS:**

10. **Voronova A.**, Coyne E., Al Madhoun A., Fair J., St-Louis C., Li G., Thurig S., Wiper-Bergeron N., Wallace V.A. and Skerjanc I.S. "Hedgehog signalling regulates MyoD expression and activity" **JBC**, In revision
11. Al Madhoun A., Ryan T., **Voronova A.**, McIntire C., and Skerjanc I.S. "Testosterone enhances cardiomyogenesis in stem cells and recruits AR to MEF2C and HCN4 genes" **J. Mol. Cell. Card.**, In revision
12. Wong J., Mehta V., Coutu J., **Voronova A.**, Ryan T., Shelton M., and Ilona S. Skerjanc "ß-catenin is essential for efficient in vitro skeletal muscle progenitor formation but can be partially compensated by retinoic acid signaling", **PLoS ONE**, In revision

**IN PREPARATION REVIEWS:**

13. **Voronova A.,** and Skerjanc I.S. "From embryonic stem cell skeletal myogenesis to future stem cell therapies"

**PUBLISHED ABSTRACTS:**

Oral presentations:

1. **Voronova A.**, Fischer A., Ryan T., Al Madhoun A., and Skerjanc I.S. "Ascl1/Mash1 is a novel target of Gli2 during Gli2-induced neurogenesis in P19 EC cells", BMI (Biochemistry, Microbiology and Immunology) seminar day, 2011, Ottawa, Canada. ***2nd prize award***
2. **Voronova A.**, Fischer A., Al-Madhoun A., and Skerjanc I. S. "The role of Gli2 in stem cell lineage specification", Concordia University's Chemistry and Biochemistry Graduate Research Conference, 2009, Montreal, Canada.

Poster presentations:

3. **Voronova A.**, Coyne E., Al Madhoun A., Fair J., St-Louis C., Li G., Thurig S., Wiper-Bergeron N., Wallace V.A. and Skerjanc I.S. "Hedgehog signalling regulates MyoD expression and activity". Development, Function and Repair of the Muscle Cell Symposium, 2012, New York City, USA
4. Coyne, E., **Voronova A**., and Skerjanc I.S. "Regulation of MyoD expression by Glioma Associated Factor 2" Undergraduate student poster competition, 2012, University of Ottawa, Canada ***Poster award***
5. Skerjanc I.S., Al Madhoun A., Ryan T., **Voronova A.**, McIntire C. and Ruel M. "Testosterone Enhances cardiomyogenesis by directly upregulating MEF2C and HCN4" Cardiovascular Development and Regeneration Symposium, 2012, New Mexico, USA
6. Fair J., **Voronova A.** and Skerjanc I. S. "Regulation of cardiomyogenesis in mouse embryonic stem cells by Glioma Associated Factor 2" International Society for Stem Cell Research (ISSCR) 9th Annual Meeting 2011, Toronto, Canada
7. **Voronova A.,** Fischer A., Ryan T., Al Madhoun A. and Skerjanc I. S. "Mash1 is a novel direct target of Gli2 during Gli2-induced neurogenesis in murine P19 embryonal

carcinoma stem cells" International Society for Stem Cell Research (ISSCR) 9th Annual Meeting 2011, Toronto, Canada

8. **Voronova A.**, Al-Madhoun A., Fischer A., Shelton M., Karamboulas C. and Skerjanc I. S. "Gli2 and MEF2C activate each other's expression and function synergistically during myogenesis in vitro" EMBO Myogenesis Conference Series "The Molecular and Cellular Mechanisms, Regulating Skeletal Muscle, Development and Regeneration" 2011, Wiesbaden, Germany
9. **Voronova A.**, Al-Madhoun A., Fischer A., Shelton M., Karamboulas C. and Skerjanc I. S. "Gli2 and MEF2C activate each other's expression and function synergistically during cardiac myogenesis in vitro" Ottawa Cardiovascular Symposium, 2011, Ottawa, Canada. ***Poster award***
10. Porter T., **Voronova A.,** Fischer A., Al Madhoun A., and Skerjanc I.S. "Gli2 induces neurogenesis in P19 EC cells via direct upregulation of Ascl1/Mash1" Neuroscience 40th Annual Meeting, 2010, San Diego, USA.
11. **Voronova A.**, Al-Madhoun A., Fischer A., and Skerjanc I. S. "Gli2 and MEF2C interact on genetic and protein levels during stem cell differentiation", Weinstein Cardiovascular Development Conference, 2010, Amsterdam, Netherlands. ***Travel award***
12. **Voronova A.**, Al-Madhoun A., Fischer A., and Skerjanc I. S. "Understanding morphogenic properties of Gli2 during stem cell differentiation"**,** The Ottawa Conference on New Directions in Biology and Disease of Skeletal Muscle, 2010, Ottawa, Canada
13. Avdic V., **Voronova A**., Tremblay V., Skerjanc I. and Couture J. F. "Inhibition of muscle cell differentiation by a peptide tailored for the high affinity binding of WDR5" Chromatin: Structure & Function Conference, 2009, Guanacaste, the Republic of Costa Rica.
14. **Voronova A.**, Skerjanc I. "Regulation of stem cell differentiation programs by Shh signaling", CIHR National poster competition, 2009, Winnipeg, Canada. ***"Honorable mention" award.***
15. **Voronova A.,** Skerjanc I. "Characterization of Gli2 transcription factor in stem cell differentiation programs", ICHR's Young Investigators Forum, 2009, Ottawa, Canada.
16. **Voronova A.,** Skerjanc I. "Searching for interactions of Gli2 in stem cell differentiation programs", Progress in Systems Biology: the Brain and Mind, 2009, Ottawa, Canada. ***"Best poster" award.***
17. **Voronova A.,** Skerjanc I. "The effect of Gli2 on stem cell differentiation programs", Cardiac Disease: Development, Regeneration, and Repair Symposium, 2009, Asheville, USA.
18. Wan S., Staehler J., Mottiar Y., **Voronova A.**, Zaidi M., and Altosaar I. "Atmosphere phytoremediation of nitrous oxide: expression of nitrous oxide reductase from *Pseudomonas Stutzeri* in transgenic plants", Green Crop Network Annual General Meeting 2007, Ottawa, Canada.

**19.** Palumaa P., **Voronova A**., Kangur L., Sillard R., Meyer-Klaucke W., Meyer T., Rompel A., "Mammalian copper chaperone Cox17 exists in two metalloforms, linked by oxidative switch" Abstract number: G2-098P Budapest, Hungary, 2005.

**INTELLECTUAL PROPERTY:**

Pill K., Sadam H., Smirnova J., **Voronova A.**, Zovo K. Registered utility model EE 00629 U1: "Multi-purpose attachable holder panels empowered with suction cups on both sides" 2006.