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Study of the molecular mechanisms involved in cell differentiation of embryonic stem cells

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Study of the molecular mechanisms involved in cell differentiation of embryonic stem cells

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A Claudia y Diego
Ao Nuno

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Y, ¡qué viva México, c...!

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Abbreviations and Acronyms

BiFC	Bi-molecular fluorescence complementation
CBP	cAMP-response-element binding protein
CC	Cardiac crescent
ChIP	Chromatin immunoprecipitation
CITED2	CBP/p300-interacting transactivator with ED-rich tail 2
CNC	Cardiac neural crest
CP	Cardiac progenitors
E	Embryonic day
EB	Embryoid body
ESC	Embryonic stem cell
FHF	First heart field
GATA4	GATA binding protein 4
GSK	Glycogen synthase kinase
HD	Hanging drop (method)
hES	Human embryonic stem (cell)
hiPS	Human induced pluripotent stem (cell)
iPS	Induced pluripotent stem (cell)
Isl1	Islet-1 transcription factor, LIM/homeodomain
LIF	Leukemia inhibitory factor
LPM	Lateral plate mesoderm
MCP	Multipotent cardiovascular progenitors
MEF	Mouse embryonic fibroblast
MEF2c	Myocyte enhancer factor 2C
mES	Mouse embryonic stem cell
Mesp1	Mesoderm posterior protein 1
NCC	Neural crest cells
Nkx2.5	Nk2 transcription factor related, locus 5
Oct-4	Octamer-binding transcription factor 4
PS	Primitive steak
SHF	Second heart field
srj	Serine-glycine rich junction
Tbx5	T-box transcription factor 5

Abstract

Potential heart progenitors from postnatal and adult heart have been identified in rodents and humans, and were shown to differentiate *in vitro* into mature cardiomyocytes. However, in some studies only immature cardiac cells were obtained, leading to concerns about whether the inefficient cell electrical maturation and electrical coupling of derivative cells will cause arrhythmias upon cellular therapies of injured hearts. Notwithstanding these concerns, recent clinical assays have indicated an improvement of heart function and life quality of patients treated with autologous cardiac progenitor cells. With the discovery of the capacity of embryonic stem cells (ESC) to differentiate into all lineages, including the cardiovascular lineage, many researchers have been focusing on developing strategies to efficiently direct ESC differentiation for the production of unlimited numbers of cardiomyocytes. Yet, the low efficiency of cardiac differentiation, its teratogenic nature and the lack of reliable protocols to purify the desired cells from ESC cultures are holding back their use for clinical application. Hence, understanding the molecular mechanisms that regulate commitment and differentiation of diverse muscle and non-muscle cell lineages of the heart is essential to improve current therapies and to design novel heart stem cell-based therapeutic strategies for cardiac regenerative medicine.

Many transcription factors, including Nkx2.5, Gata4 and Isl1, have been described as master regulators of cardiac morphogenesis. Indeed, they are required for the commitment and differentiation of cardiac progenitors in early embryonic development, as well as for the differentiation of ESC into cardiovascular lineages. Furthermore, mutations in these genes have been associated with congenital heart defects in humans. This finding underscores the clinical relevance of studying the role of cardiac transcription factors during heart development.

Cited2 is a transcriptional regulator essential for mouse embryonic development. Amongst a wide spectrum of developmental defects presented by Cited2 knockout mouse embryos, the cardiovascular and left-right patterning defects are the most prominent. Importantly, heterozygous mutations of human CITED2 in association with congenital heart disease have been reported by several research groups. Moreover, it has been suggested that Cited2 is important for ESC-derived cardiovascular

development and essential in the epiblast or its derivatives for normal cardiac left-right patterning. Although Cited2 was shown to be required for the maintenance of pluripotency and self-renewal of ESC, its role in early embryonic development and its mechanism of action are still not well explored.

In the present study, we investigated the role of Cited2 in mouse ESC differentiation towards cardiac cells. By performing increasing and absence of Cited2 expression in ESC, we have established that Cited2 is essential for cell commitment and differentiation towards the mesoderm, ectoderm and endoderm lineages. Indeed, Cited2 depletion impaired ESC differentiation to several cell lineages. More importantly, Cited2 was required for the normal emergence of mesoderm cells from early ESC specification to cardiac lineages. Our results suggest that this response is primarily associated with the direct or indirect modulation of *Nodal* and *Brachyury* gene expression during mesodermal cell commitment, thus enabling a signaling cascade promoting cardiogenesis. Additionally, our results indicated that loss of Cited2 generated comparatively less cardiomyocytes in differentiated ESC cultures, whereas gain of Cited2 led to higher numbers of cardiomyocytes. These findings suggest Cited2 is an important protein for cardiac function. Furthermore, our data suggest that Cited2 can modulate the gene expression (Islet1, Gata4, Nkx2.5 and Tbx5) in cardiac progenitors, thus playing an important role during cardiac cell commitment. We have also found that Cited2 plays a role in cardiomyocyte proliferation, as indicated by the downregulation of Tbx5 and its targets genes upon Cited2 depletion. Furthermore, by overexpressing human CITED2 and Gata4 in murine ESC, we found that Cited2 could not prevent endoderm commitment induced by Gata4 overexpression. The same overexpression approach was used to test the role of CITED2-ISLET1 interaction in cardiac differentiation, and the results indicate that the cardiogenic response of Cited2 might be additive in association with that induced by Islet1 (Isl1) overexpression.

We have also found Cited2 expression in multipotent cardiac progenitor population, specifically in secondary heart field (SHF) progenitors, which are marked by the expression of Isl1 protein. Our data generated by increasing and absence of Cited2 Cited2 expression in SHF progenitor, suggest that finely regulated Cited2 gene expression levels could allow or disrupt the SHF-differentiation program. Moreover, by performing GST-pull down and by bi-molecular fluorescence complementation assays, we gathered evidence indicating that CITED2 physically interacts with ISL1. By

performing chromatin immunoprecipitation assays we have also found that CITED2 binds the Isl1 promoter region.

Taken together, our results indicate that Cited2 is important for proper cardiovascular differentiation by acting at different levels during cardiac stem cell commitment: firstly, by inducing ESC towards mesoderm at early events during ESC commitment; and secondly, by inducing and maintaining the expression of cardiac transcription factors important for cardiogenesis. Although many Cited2 functions remain uncovered, our data indicate that Cited2 is an important cardiac differentiation regulator that can participate in heart regeneration, and therefore be useful for the development of alternative approaches to treat or prevent heart failure.

Sumário

Potenciais progenitores cardíacos de coração pós-natal e adulto foram identificados em roedores e seres humanos, e verificou-se serem capazes de se diferenciar *in vitro* em cardiomiócitos maduros. No entanto, em alguns estudos foram obtidos apenas células cardíacas imaturas, assim suscitando questões sobre se ineficiente maturação elétrica celular e acoplamento eléctrico das células derivadas não causará arritmias no contexto da aplicação de terapias celulares em lesões cardíacas. Apesar destas questões, ensaios clínicos recentes indicaram uma melhoria da função cardíaca e da qualidade de vida dos pacientes tratados com células progenitoras cardíacas autólogas. Com a descoberta da capacidade de células estaminais embrionárias (ESC) se diferenciarem em todas as linhagens, incluindo a linhagem cardiovascular, muitos investigadores se têm concentrado no desenvolvimento de estratégias para dirigir eficientemente a diferenciação de ESC para a produção de um número ilimitado de cardiomiócitos. No entanto, a baixa eficiência da diferenciação cardíaca, a sua natureza teratogénica e a falta de protocolos bem validados para purificar as células desejadas das culturas de ESC estão a atrasar o seu uso para aplicações clínicas. Assim, a compreensão dos mecanismos moleculares que regulam o comprometimento e diferenciação de diversas linhagens de células musculares e não-musculares do coração é essencial para melhorar as terapias atuais e para conceber novas estratégias terapêuticas baseadas em células estaminais para a medicina regenerativa cardíaca.

Muitos factores de transcrição, incluindo Nkx2.5, Gata4 e ISL1, têm sido descritos como reguladores importantes da morfogénese cardíaca. Com efeito, eles são necessários para o comprometimento e diferenciação de células progenitoras cardíacas no desenvolvimento embrionário inicial, assim como para a diferenciação das ESC em linhagens cardiovasculares. Além disso, mutações nestes genes foram associadas a doenças cardíacas congénitas em pacientes humanos. Esta observação reforça a relevância clínica do estudo do papel dos fatores de transcrição cardíacos no desenvolvimento do coração.

O Cited2 é um regulador de transcrição essencial para o desenvolvimento embrionário murino. Entre um amplo espetro de defeitos de desenvolvimento

apresentados por embriões de ratinho Cited2 knockout, defeitos cardiovasculares e de padronização esquerda-direita são os mais proeminentes. É importante ressaltar que mutações heterozigóticas de CITED2 humano em associação com cardiopatias congénitas têm sido relatados por vários grupos de investigação. Além disso, tem sido sugerido que Cited2 é importante para o desenvolvimento cardiovascular derivado de ESC e é essencial no epiblasto ou seus derivados para a padronização esquerda-direita cardíaca. Embora o Cited2 seja necessário para a manutenção da pluripotência e auto-renovação de ESC, o seu papel no desenvolvimento embrionário precoce e o seu mecanismo de ação ainda não foram bem explorados.

No presente estudo, investigamos o papel de Cited2 em diferenciação de ESC de ratinho em células cardíacas. Através da realização de experiências de ganho e de perda de função de Cited2 em ESC, nós verificámos que Cited2 é essencial para o comprometimento e diferenciação em células de linhagens de mesoderme, ectoderme e endoderme. De facto, a depleção de Cited2 perturbou a diferenciação de ESC para diversas linhagens de células. Mais importante ainda, Cited2 foi necessário para o aparecimento normal de células da mesoderme a partir da especificação precoce de ESC para linhagens cardíacas. Os nossos resultados sugerem que esta resposta está principalmente associada com a modulação direta ou indireta da expressão dos genes *Nodal* e *Brachyury* durante o comprometimento em célula mesodermal, permitindo assim uma cascata de sinalização que promove a cardiogénese. Além disso, os nossos resultados indicam que a perda de Cited2 levou à geração de menos cardiomiócitos em culturas de ESC diferenciadas, enquanto que o ganho de Cited2 levou a um maior número de cardiomiócitos. Estes resultados sugerem portanto que Cited2 é uma proteína importante para a função cardíaca. Além disso, nossos dados sugerem que Cited2 pode modular a expressão génica (Islet1, Gata4, Nkx2.5 e Tbx5) em progenitores cardíacos, desempenhando assim um papel importante durante o comprometimento de células cardíacas. Nós também descobrimos que Cited2 desempenha um papel na proliferação de cardiomiócitos, como indicado pela regulação negativa de Tbx5 e seus genes-alvo na situação de depleção de Cited2. Além disso, através de sobreexpressão de CITED2 humano e Gata4 em ESC, descobrimos que Cited2 não impede o comprometimento em endoderme induzido pela sobreexpressão de Gata4. A mesma abordagem de sobre-expressão foi usada para testar o papel da interação CITED2-ISLET1 na diferenciação cardíaca, e os resultados indicam que a resposta cardiogénica

de Cited2 será aditiva em associação com aquela induzida pela sobreexpressão de Islet1 (ISL1).

Também detetámos expressão de Cited2 em populações de progenitores cardíacos multipotentes, especificamente em progenitores do campo cardíaco secundário (SHF), que são marcados pela expressão da proteína ISL1. Os nossos dados obtidos em experiências de ganho e perda da função de Cited2 em progenitores SHF sugerem que níveis de expressão do gene Cited2 finamente regulados podem permitir ou interromper o programa de diferenciação SHF. Além disso, através da realização de ensaios de GST-pull down e ensaios de fluorescência de complementação bi-molecular, obtivemos dados indicando que CITED2 interage fisicamente com ISL1. Através da realização de ensaios de imunoprecipitação de cromatina também descobrimos que CITED2 se liga à região do promotor de ISL1.

Em conjunto, os nossos resultados indicam que Cited2 é importante para uma diferenciação cardiovascular adequada, agindo em diferentes níveis durante o comprometimento de células estaminais cardíacas: em primeiro lugar através da indução de ESC para mesoderme em estadios precoces durante o comprometimento de ESC; e em segundo lugar através da indução e manutenção da expressão de fatores de transcrição cardíacos importantes para a cardiogénese. Embora muitas funções de Cited2 permaneçam por descobrir, os nossos dados indicam que Cited2 é um importante regulador da diferenciação cardíaca, que pode participar na regeneração do coração e, portanto, ser útil para o desenvolvimento de abordagens alternativas para o tratamento ou prevenção da insuficiência cardíaca.

Resumen

A la fecha se han identificado potenciales precursores cardíacos en corazón posnatal y adulto, de roedores y seres humanos, a los que han llevado a diferenciarse en cardiomiocitos maduros *in vitro*. Sin embargo, en algunos estudios de diferenciación sólo se lograron obtener las células cardiacas inmaduras, lo que levanta preocupaciones acerca de la ineficiente maduración en células cardiacas eléctricas y su acoplamiento eléctrico, ya que han causado arritmias una vez utilizadas para terapias celulares en corazones lesionados. A pesar de estas preocupaciones, los ensayos clínicos recientes con la utilización de precursores cardíacos, han indicado una mejoría en la función cardíaca y en la calidad de vida de los pacientes tratados con células progenitoras autólogas cardíaca. Con el descubrimiento de la capacidad de las células madre embrionarias (ESC) de diferenciarse en todos los linajes, incluyendo el cardiovascular, muchos investigadores se han centrado en el desarrollo de estrategias de diferenciación eficiente y directa apartir de ESC para la producción de un número ilimitado de cardiomiocitos. Sin embargo, la baja eficiencia en la diferenciación cardiaca, su carácter teratogénico y la falta de protocolos fiables para purificar las células deseadas de las culturas del ESC continúan deteniendo su uso para la aplicación clínica. Por lo que, la comprensión de los mecanismos moleculares que regulan el compromiso y la diferenciación de las ESC en los diversos músculos y en los linajes de células no musculares del corazón es esencial para mejorar los tratamientos actuales y diseñar nuevas estrategias basadas en células madre del corazón terapéuticos de medicina regenerativa cardíaca.

Muchos factores de transcripción, incluyendo Nkx2.5, Gata4 y ISL1, han sido descritos como reguladores maestros de la morfogénesis cardíaca. Son necesarios para el compromiso y la diferenciación de los progenitores cardíacos durante el desarrollo embrionario temprano, así como para la diferenciación de ESC en linajes cardiovasculares. Además, las mutaciones en estos genes han sido asociados con defectos congénitos del corazón en los seres humanos. Este hallazgo subraya la relevancia clínica de estudiar el papel de los factores de transcripción cardiaco durante el desarrollo del corazón.

Cited2 es un regulador transcripcional esencial para el desarrollo embrionario en ratón. Entre una amplia gama de defectos presentados en embriones Cited2 de ratones knock-out, se destacan los defectos cardiovasculares y de asimetría (izquierda y derecha). Es importante subrayar que las mutaciones heterocigotas de CITED2 humano en asociación con cardiopatías congénitas han sido reportados por varios grupos de investigación. Por otra parte, se ha sugerido que Cited2 es importante para el desarrollo cardiovascular derivado de ESC y siendo Cited2 esencial en el epiblasto o sus derivados para el patrón izquierda-derecha cardíaco normal. Aunque Cited2 ha demostrado ser necesario para el mantenimiento de la pluripotencia y auto-renovación del ESC, su papel en el desarrollo embrionario temprano y su mecanismo de acción no es aún bien explorado.

En el presente estudio, se investigó el papel de Cited2 en la diferenciación de ESC en células cardíacas. Mediante la realización de ganancia y pérdida de función de la proteína Cited2 en ESC, hemos establecido que Cited2 es esencial para el compromiso y la diferenciación celular hacia los linajes mesodermo, ectodermo y endodermo. De hecho, la pérdida de Cited2 afectada la diferenciación de ESC para varios linajes de células. Más importante aún, Cited2 es necesario para la aparición normal de las células del mesodermo de la especificación temprana de las ESC para linajes cardiacos. Nuestros resultados sugieren que esta respuesta está asociada principalmente con la modulación directa o indirecta de Nodal y Brachyury la expresión génica durante el compromiso de células mesodérmico, permitiendo así una cascada de señalización promover la cardiogénesis.

Además, nuestros resultados indican que la pérdida de Cited2 genero comparativamente menos cardiomiocitos en ESC culturas diferenciadas, mientras que la ganancia de Cited2 condujo a un mayor número de cardiomiocitos. Estos hallazgos sugieren Cited2 es una proteína importante para la función cardíaca. Además, nuestros datos sugieren que Cited2 puede modular la expresión génica (Islet1 , Gata4 , Nkx2.5 y Tbx5) en progenitores cardíacos, jugando así un papel importante durante el compromiso de las células cardíacas. También hemos encontrado que Cited2 juega un papel en la proliferación de los cardiomiocitos, como se indica por la regulación a la baja de Tbx5 y sus objetivos de los genes durante la pérdida de Cited2. Además, mediante la sobreexpresión de CITED2 humana y Gata4 en ESC , encontramos que Cited2 no pudo evitar el compromiso endodermo inducida por la sobreexpresión Gata4. El mismo

enfoque sobreexpresión se utiliza para probar el papel de la interacción CITED2 - ISLET1 en la diferenciación cardiaca, y los resultados indican que la respuesta cardiogénico de Cited2 podría ser aditivo en asociación con la inducida por la sobreexpresión de Islet1 (ISL1).

También hemos encontrado la expresión génica de Cited2 en multipotentes progenitoras población cardíaco, especialmente en el campo del corazón secundaria (SHF) progenitores, que se caracterizan por la expresión de la proteína ISL1. Nuestros datos generados por la ganancia y pérdida de función en Cited2 SHF progenitor, sugieren que los niveles de expresión de genes Cited2 reguladas finamente podrían permitir o interrumpir el programa de SHF-diferenciación. Por otra parte, mediante la realización de GST-pull downs y por ensayos de complementación de fluorescencia bi-molecular, reunimos evidencia que indica que CITED2 interacciona físicamente con ISL1. Mediante la realización de ensayos de inmunoprecipitación de la cromatina también hemos encontrado que CITED2 se une la región del promotor ISL1.

En conjunto, nuestros resultados indican que Cited2 es importante para la diferenciación cardiovascular actuando en diferentes niveles durante el compromiso cardiaco en células madre: en primer lugar, mediante la inducción de ESC hacia mesodermo en eventos tempranos; y en segundo lugar, mediante la inducción y el mantenimiento de la expresión de factores de transcripción cardiacos importantes para cardiogénesis. Aunque aún muchas de las funciones Cited2 permanecen sin descubrir, nuestros datos indican que Cited2 es un importante regulador de la diferenciación cardiaca que pueden participar en la regeneración del corazón, y por tanto ser útil para el desarrollo de enfoques alternativos para tratar o prevenir la insuficiencia cardiaca.

CHAPTER I

INTRODUCTION

1. Introduction

1.1. Embryonic stem cells

The first definition of embryonic stem cells (ESC) was made by LeRoy Stevens in 1954, when ovarian teratomas cultures *in vitro* were defined as a "Pluripotential embryonic cells appear to give rise to both rapidly differentiating cells and others which like themselves, remain undifferentiated"[1]. In the same decade, Barry Pierce demonstrated that a transplantation of a single cell embryonal carcinoma (EC) *in vivo* resulted in the formation of a teratocarcinoma[1]. Experimental studies of EC cells biology and properties laid the foundation for the concepts of embryonic cells with pluripotential differentiation capacities and able to self-renew[1]. The experimental culture conditions established for EC cells were the basis for the culture conditions that lead to the successful isolation of pluripotent mouse embryonic stem cells (mESC) in 1981 from the inner cell mass of a delayed blastocyst[2]. The mESC were established and cultured with conditioned media[3], characterized and tested for their ability to contribute to the development of a chimeric mouse[4]. The variable conditioned media was replaced by the culture of mESC on feeder layers constituted by inactivated mouse embryonic fibroblast (MEF) and by activation of leukemia inhibitory factor (LIF)-STAT3 signaling pathway, which can robustly sustain the self-renewal of undifferentiated mESC[5]. Moreover, studies conducted on ESC established the ability of these cells to originate cells of the three germ layers and to contribute to the development of a chimeric embryo when injected in a host blastocyst, which was subsequently re-implanted into a pseudo-pregnant female.

The establishment of the ESC-based technology opened the possibility to explore mammalian genetics *in vivo*, and also to understand some developmental molecular mechanisms *in vitro*, since ESC differentiation processes are controlled by signaling pathways and key proteins that also involved in embryonic development[6]. Of course, the following question was if this technology could be applied for human embryos. Although this rose a wave of public and academic concern, due to intrinsic ethic issues (i.e. cloning a human being); in 1998 a paper was published demonstrating the isolation

of human embryonic stem cells (hESC)[7], where the fibroblast growth factor 2 (FGF2) is crucial for renewal and maintenance of the pluripotency state[8].

1.2. Induced pluripotent stem cells

In 2006, a new technology was presented, in which the forced exogenous expression in mouse embryonic or adult fibroblast cultures enables some of the somatic cells to acquire a pluripotent state, and these newly reprogrammed cells were named "induced pluripotent stem" (iPS) cells[9]. The iPS cells have properties similar to those of the ESC counterparts, including the ability to generate viable and fertile adult mice chimeras when implanted in a host blastocyst[10]. The four reprogramming transcription factors were selected amongst a set of proteins known for their critical role in the maintenance of pluripotency, cell phenotype and proliferation in early embryos, as well as in ESCs. The "fantastic" four transcription factors, Oct4, Sox2, c-Myc and Klf4, are also known as the Yamanaka's factors, as a tribute to Professor Shinya Yamanaka who established this new technology. Although, other combinations of transcription factors and other proteins have now been successfully used to reprogram somatic cells by many research groups worldwide, Oct4, Sox2, c-Myc and Klf4 remain the most classically used combination.

In 2007, the same group led by Shinya Yamanaka, generated iPS cells derived from human dermal fibroblast and other human somatic cells by using the same four transcription factors [11]. The human iPS (hiPS) cells are similar to hESC in many aspects, such as the morphology, proliferation, differentiation potential *in vitro*, and in their ability to form teratomas, which is a hallmark of pluripotent cells. Although the use of human embryos faces ethical controversies, the potential of hiPS cells opened the possibility to generate patient-disease-specific pluripotent stem cells for medical applications, as well as for the better understanding disease mechanism, drug screening and toxicology applications. This achievement was a breakthrough for biology, genetics and medicine due to the unlimited resource of human cells that have the potential to differentiate into a specific germ line, leading a new era for human cell regeneration.

In fact, in 2013 several companies and research groups have announced the current development of clinical trials based on hiPS-derived cells.

1.2.1. Differentiation

The cellular differentiation is defined as an acquisition of cell specialization, occurring in a multi-step and time regulated process, which starts from commitment and the determination of cell fate.

It is well known that the potential of ESC for regenerative therapies resides in their capacity to differentiate *in vitro* and undergo terminal differentiation into cells derived from mesoderm, ectoderm and endoderm lineages[12]. During the 70's decade the emerging area of establishment of new cell cultures and the isolation of ESC lineages, led to the observation that isolated cells have a differentiation potential, and they followed the normal pathways of development, and the first cell types emerging from differentiation were like primary extra-embryonic endonderm[1]. In 1985, an *in vitro* model based on differentiating ESCs was established and clearly demonstrated the potential of ESCs to give rise to cells of the three germ layers[13].

Multiple research groups have worked and established protocols to promote the efficient and reproducible differentiation of ESCs into cell types of interest *in vitro*. The first step of differentiation, which is common to most, if not to all, the protocols is the removal of stimulus needed for self-renewal and maintenance of pluripotency of ESCs (i.e., removal from the culture conditions LIF, FGF2 or feeder cells).

After withdrawal from the culture of signaling molecules maintaining ESC pluripotent, several strategies have been implemented to achieve the complete differentiation of ESC into the desired cells types. The following are the basic strategies to induce *in vitro* differentiation of ESC:

- Embryoid body (EB) formation is referred as the change in the culture conditions from two-dimensional monolayer cell cultures to three-dimensional cell-based structures[13]. Briefly, ESCs have to be dissociated from colonies and transferred into suspensions cultures, in which ESCs are allowed to aggregate and form spherical three-dimensional structures called embryoid bodies. The removal of molecules promoting self-renewal from the culture medium combined with cellular aggregation is an efficient way to differentiate ESC, in a

mixture of cells derived from the three germ layers, including mature cardiac myocytes with autonomous and spontaneous contractile abilities.

- Directed differentiation is implemented to overcome the heterogeneous cell mixture derived from EBs differentiation cultures. The directed differentiation is achieved by controlling the nutrient intake, the extracellular matrix (ECM), co-culture with inductive cell types or the addition of a signaling molecule that have an impact on gene expression and cell proliferation.
- Genetic manipulation of ESCs to force the expression of some transcription factors that can direct differentiation towards a specific lineage. GATA4 exogenous expression, for example, promotes the differentiation of ES cells into endoderm[14], and in combination with other transcription factors Gata4 promotes cell differentiation into cardiac cells [15].

1.2.2. Embryoid body formation

EB formation has been utilized widely as a trigger of *in vitro* differentiation[16] from both mouse[13] and human[12] ESC. The resultant three-dimensional cell disposition facilitates multicellular interactions; in which cell-cell contact exists and gap junctions may be established[16]. The EB aggregates resulting from mESC present distinct cell differentiation process depending on the region of the EB, and further developing into a embryonically distinct cell types[13]; whereas in human EB-derived aggregates display a more random organization[12].

During EB maturation, which normally takes 2-6 days, ESCs suffer enormous morphological changes[13], and acquire several molecular markers of differentiated cells types[12]. The quality of formed EBs affects the subsequent differentiation[12].

The hanging drop (HD) culture is the most frequently technique used to form EBs. This method consists in maintaining a known quantity of ESCs into a small drop; this drop is placed on the inverted petri dish lid. Each drop hangs by surface tension and the ESCs sediment by gravity to the bottom of the drop. Typically the EB maturation takes place in suspension culture using bacterial-grade dishes.

Although, a report claimed that the differentiation of EBs formed by the HD method favors preferentially mesodermal cell lineages[11], EBs have been used to generate a broad spectrum of cell types, including neuronal cells, lymphoid, hematopoietic cells,

cardiomyocytes, smooth muscle cells, chondrocytes, renal cells, insulin-producing cells and gametes. However, the HD method presents disadvantages such as the limited liquid working volume (<50 μL) used to form a drop, the impossibility to exchange the culture medium of a drop and the inability to perform direct microscopic observations.

To overcome the limitation of EB size heterogeneity, a forced aggregation protocol using V- or U- shaped low-adherence 96-well plates has been employed[17]. This method is quite reproducible, practical and efficient for aggregation-based differentiation, and makes possible to perform microscopic observations of a particular EB selected during the cultivation. As in HD method, EB formation using low-adherence vessels can be constituted of a predetermined number of ESCs per well.

Although EB suspension and aggregation methods are commonly used, they are technically complex and time consuming, which has led to the development of monolayer differentiation based methods[18].

1.2.3. Directed differentiation

The main focus of the directed differentiation is to avoid the generation of heterogeneous mixture of specialized cells, which typically happens during a non-directed differentiation process, such as the HD method[19]. The directed differentiations normally achieve high efficiency rates of differentiation towards a unique cell type of interest. Although the first stage of differentiation for all cell types normally includes the EB formation, the specification stage takes place by replacing the media in which EBs where seeded and, adding one or several growth factors or molecules promoting differentiation, depending the cell type needed.

The boost of differentiation toward a specific lineage can be achieved by different mechanism, such as the activation of endogenous transcription factors, transfection of ESCs with vectors ubiquitously expressing transcription factors, exposition of ESCs to selected growth factors or co-culturing ESCs with cell types capable of lineage induction[20]. Typical growth factors such asactivin-A, basic fibroblast growth factor (bFGF), bone morphogenic protein (BMP) -4, hepatocyte growth factor, retinoic acid (RA) and transforming growth factor β (TGFβ), among others are often applied to ESC cultures.

For example, formation of ectodermal derivatives from ESCs can be achieved by adding RA, and the stimulation of sonic hedgehog (SHH) pathways and TGFβ families[21]. Moreover, oligodendrocytes, dopaminergic neurons and motor neurons, can be originated *in vitro* by adding members of the bFGF/EGF family proteins to ESC differentiated cultures. Enrichment of hepatocytes in ESC differentiated cultures can be obtained (up to 70%) by adding activin A, FGF2, a deleted variant of hepatocyte growth factor and dexamethasone in the culture medium[22]. Moreover, the addition of RA after the EB formation enhances the expression of cardiac-specific genes, resulting into ventricular cardiomyocytes[23].

Since ES cells fate upon differentiation depends on the complex interaction between growth factors, signaling molecules and the ECM proteins constituting the ESCs enviromental niche, the use of ECM in directed differentiation takes advantage of this fact. The interaction between ESCs-derived cells and ECM via integrins determines the expression of signaling molecules that affect ESC differentiation[24-26]. The ECM and integrins collaborate to regulate gene expression associated with cell growth, differentiation and survival.

These studies established the importance of exposure of ESC to various stimuli at specific times and at specific doses that are essential for directing differentiation towards more specific cell fates. Although, some important aspects to manage ESC *in vitro* differentiation into a some specific cell type (i.e. emerging time of subtype cell fates, its regulation, which signals are using) are known, the reliable conditions for the majority of the cells types are still largely unknown or remain to be establish

1.2.4. Trans-differentiation

The generation of iPS cells demonstrated that the expression of a specific combination of defined factors in somatic specialized cells alters the global gene expression of these cells, leading them to acquire novel plasticity and functions. Moreover, it has been known for a long time that some transcription factors are master regulators of differentiation or developmental programs, and their ectopic expression in certain cell type contexts will trigger these programs. The idea that these master regulators might also have the intrinsic capacity of reprogramming somatic cells with a specific function into cells with a different and related to the function of cells that these

regulators might also have the intrinsic capacity of reprogramming somatic cells with a specific function into cells with a different function related to those of the cells that these master regulators normally contribute to develop, has now been tested in several contexts. Indeed, the bHLH transcription factor, Neurogenin 3, in combination with Pdx1 and Mafa, can reprogram pancreatic exocrine cells into functional β cells *in vivo* [27]. A combination of three factors, Ascl1, Brn2, and Myt1l, switches dermal fibroblasts to functional neurons [28]. Gata4, Mef2c and Tbx5 trans-differentiated postnatal cardiac or dermal fibroblast directly into cardiomyocyte-like cells without going through a progenitor stage[15]. The directed conversion of human fibroblast to multilineage blood progenitors has been achieved by selection of $CD45^+$ cells from human fibroblast cultures ectopically expressing OCT4 and maintained in culture in the presence of hematopoietic cytokines[29].

The novel approaches allowing the reconversion of cell functions and specialization from somatic cells have offered an entirely new potential source of cells for future regenerative therapies, although there is still a need for further studies in human cell processes.

1.3. *Cardiac differentiation from pluripotent stem cells*

Four main steps are required to generate cardiomyocytes from ESCs. The first step is defined by the formation of mesoendoderm derivatives that are composed of precursor cells of both the mesoderm and endoderm germ layers. The patterning of mesoderm derived from mesoendodermal precursors to a cardiogenic-mesoderm, from where the cardiac mesoderm derives. The cardiac mesoderm will give rise to two populations of cells forming the first and second heart field (FHF and SHF, respectively), that later will contribute to form all the cells of the heart; and they will continue their differentiated path until they reach the stage of maturation of early cardiomyocytes (Figure 1.1). These steps are characterized by the specific expression of several transcription factors, such as Brachyury for the primitive streak mesoderm, Mesp1 for the cardiogenic mesoderm, and Nkx2.5, Tbx5/20, Gata4, Mef2c, Islet1 (Isl1) Hand1/2 for the cardiac mesoderm derivatives.

Mature cardiomyocytes are characterized by the expression of cardiac structural proteins and proteins involved in the contractile function, such as α-actinin, a-myosin heavy chain (αMHC) or the cardiac isoform of Troponin-T (Tnt).

An intricate and coordinated action of several signaling pathways and growth factors, such as wingless (Wnt), Nodal, BMPs and FGFs amongst others, is necessary for the progress of ESC differentiation to specialized cardiac subtypes (Figure 1.2).

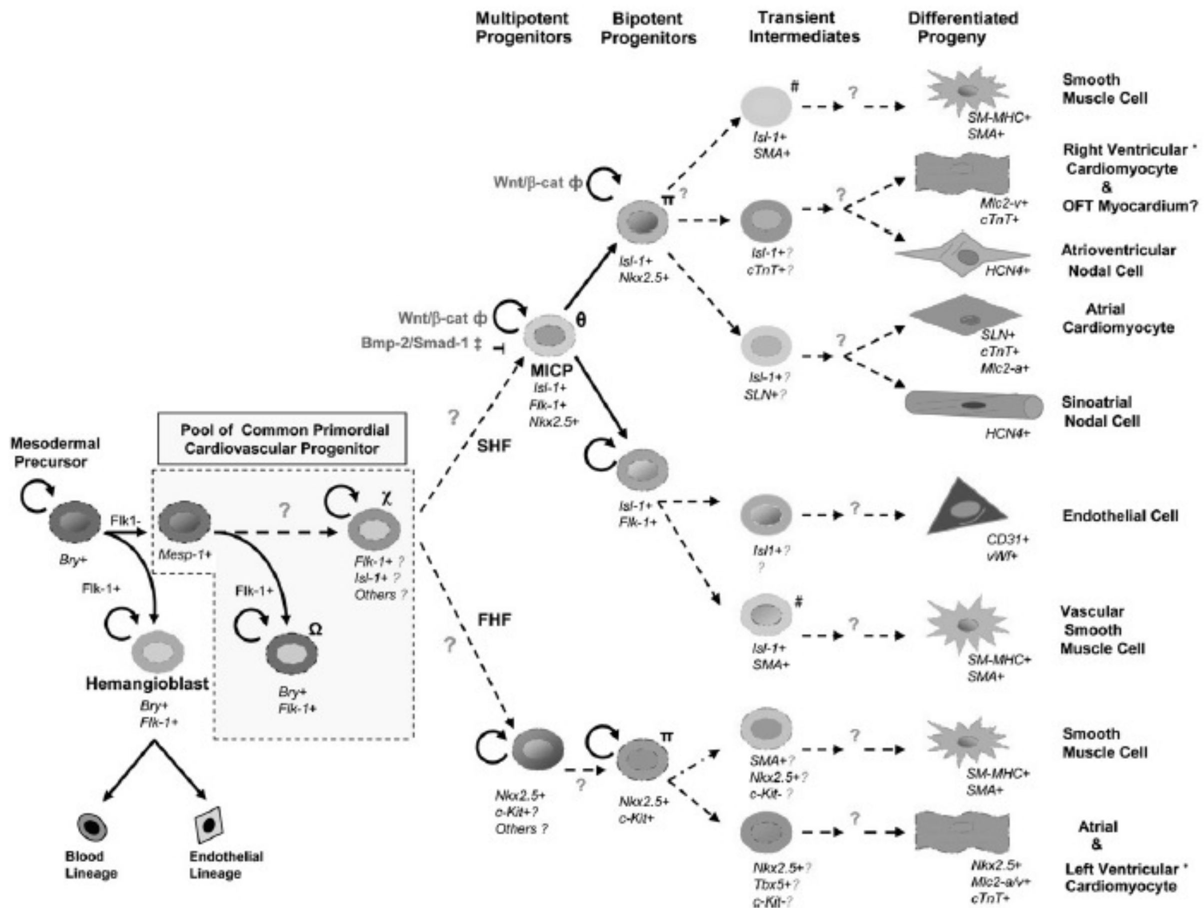


Figure 1.1. Cellular hierarchy from multipotent mesodermal progenitors to specified heart cells. Schematic diagram of heart cell lineage diversification starting with common primordial cardiovascular progenitor population (expressing Brachyury^{+}, *Bry*$^{+}$ as a marker). This progenitors wich give rise to a pool of cardiovascular progenitors that later will become the first (FHF) and second heart field progenitors (SHF), and originate all cells in the heart. Adapted from Martin-Puig *et al.* 2008[30].

1.3.1. Embryoid bodies

Cardiomyocytes are one of the first cell types derived from ESC using the EBs formation method[31] which remains a method widely applied to induce cardiomyocyte differentiation due to its simplicity and low cost. Typically, after the initial days of EB aggregation, those cells are plated on a matrix-coated tissue culture plate for further

differentiation, and within a few days after platting, contracting foci with cardiomyocytes characteristics can be observed[32].

The EB formation was the first method used for the characterization of hESC differentiation potential, the process involved the suspension of hESC colonies in media containing 20% FBS[32]. Although the formation of EB was occurring in a similar manner and a time scale comparable to those of the EB formed by mESC, the human EB did not present an organized spatial differentiation mimicking organogenesis[12], and only a minority of contracting foci expressing α-cardiac actin (embryonic myocardial cell marker) were observed in human EB.

Cardiomyocyte differentiation can also be achieved from hiPS cells in an EB-based differentiation system[33]. This methology can give rise to rhythmically contracting areas that reach up to 15% of total culture area. Also, the cardiomyocytes obtained were capable to generate a functional cardiac syncytium and the derived cardiac tissue exhibits functional electrophysiological properties.

Centrifugation of hESC into a round-bottomed ultra-low attachment 96-well plate allowed a better control of the embryoid body size, and using activin A and FGF2 produced 23% contracting EBs[17]. The same method performed under hypoxic conditions leads to an increase in the efficiency in cardiomyocyte differentiation[17]. Similar results were obtained in a system that combines the forced aggregation method with BMP4, Activin A, Wnt-3A, stem cell factor and vascular endothelial growth factor A (VEGFA) during early days of differentiation, obtaining high differentiation efficiency[34].

1.3.2. Directed differentiation

The signaling pathways regulating the cardiogenesis can be recapitulated in stem cells cultures by the addition of specific growth factors such as FGF, BMPs, WNTs (Figure 1.2), and mESC and hESC lines require optimization of these signaling pathways for efficient cardiac differentiation[35]. Several studies have shown that combinations of BMP4, Wnt-3a and Activin A, induce gastrulation-like events and mesoderm/endonderm development from pluripotent SC[36]. Both hESC[18] and iPS cells [9] have been differentiated in these conditions and yielded in 30% of cardiomyocytes amongst the cell types obtained, using a high-density stem cell culture and treated with a high dose of Activin A followed by BMP4 treatment. Improvements have been made to

this protocol to increase cardiac cell fate, such as the addition of Wnt-3A and Dickkopf Wnt signaling pathway inhibitor 1 (Dkk1), the use of matrigel to enhance EMT, removal of insulin, addition of FGF2 and Dkk1[36].

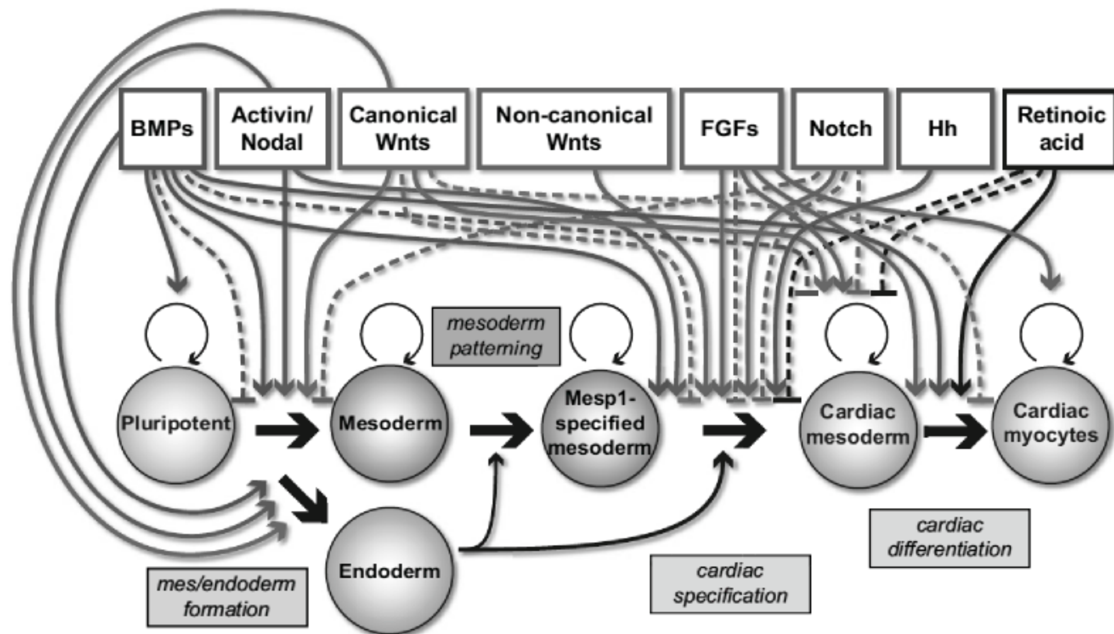


Figure 1.2. Signaling pathways involved in cardiac differentiation from pluripotent stem cells.
Schematic diagram of the main stages of differentiation from pluriptent stem cells to cardiac myocytes. Solid and dashed lines respectively specify the positive and negative action of signalling pathways at the indicated differentiation steps; circular arrows indicate self-renewal. Adapted from Noseda *et al.* 2011[37].

The canonical Wnt signaling is required for the induction of the mesoderm differentiation from ESCs, but must be inhibited after for the induction of cardiogenic mesoderm[38-40]. Respecting the biphasic activation of Wnt pathway led stem cells to differentiate and 40-50% of the differentiated cells were cardiomyocytes. Wnt-3A applied at initial steps of EB formation induced successfully mesoderm formation from hESCs[41].

Moreover, the growth factors necessary for hESCs differentiation can be provided from co-cultured mouse visceral endonderm-like cells (END-2). A further improvement in cardiomyocyte differentiation efficiency was achieved by removing fetal bovine serum (FBS) of the culture medium and by adding L-ascorbid acid[42]. However, the co-cultures or END-2 conditioned media differentiation is relative inefficient but generates mostly ventricular-like cardiomyocytes (≈85%) among the cells differentiated, which gives hope to treat heart failure, because a myocardial infarction can destroy approximately 0.5-1 billions ventricular cardiomyocytes in few hours[43].

1.3.3. Induced cardiac reprogrammed cells

The reprograming somatic cells into pluripotency state has provided new insight into direct reprograming[9]. Successful reprograming of both mouse mesoderm and non-cardiogenic mesoderm into cardiac myocytes, was obtained by the exogenous expression of Gata4, Tbx5 and Baf60c (subunit of the Swi/Snf/like BAF chromatin remodeling complex)[44]. The direct conversion of mouse fibroblast into cardiomyocytes-like cells (iCM) was achieved by exogenous expression of Gata4, Mef2c and Tbx5[15]. By reprogramming MEFs with Oct4, Sox2 and Klf4 during 4 days and, later induce the activation of cardiac program at mid-stage of reprograming, leads to a directly conversion of MEFs into cardiomyocytes in a rapid and efficient manner[45].

By transducing MEFs cultures with the transcription factor Oct4 and culturing the cells under a defined cocktail consisting of ALK4/5/7 inhibitor (SB431542), glycogen synthase kinase 3 (GSK3) inhibitor, LSD1/KDM1 inhibitor and adenylyl cyclase activator led to the efficient conversion of fibroblast to spontaneous contracting foci that exhibit a ventricular cardiomyocyte phenotype[46].

By directing reprogramming cardiac fibroblast *in vitro* and *in vivo* into adult cardiomyocyte-like cells by the addition of Gata4, Mef2c and Tbx5[47]. This approach is a hopeful methodology to restoring cardiac functions and to treating cardiac disease, by regenerating adult heart tissue from endogenous cells.

1.3.4. Resident cardiac progenitor cells

Resident populations of cardiac progenitor cells (CPC) have been identified in postnatal hearts by studying the expression of surface markers a such as c-kit or Sca-1, by the nuclear expression of fluorescent protein, or by their ability to form multicellular spheroids[34, 43]. Also these CPC have been identified in differentiated stem cell derivatives[35, 48, 49].

The c-kit$^+$ population is clonogenic, self-renewing, and multipotent. Although, the c-kit$^+$ expression is not restricted to cardiac lineages, since it is also expressed by telocytes, the thymic epithelium and mature circulating cells (hematopoietic stem cells and mast cells)[43] the c-kit$^+$ population is able to differentiate into cardiomyocytes, smooth muscle cells and endothelial cells[50].

The Sca-1+ adult mouse cardiac stem cells were shown to differentiate into cardiomyocytes after 4 weeks of treatment with 5''-azacytidine[51]. Similarly, a subset of Sca-1+ cells can differentiate into cardiomyocytes by co-culture with bulk populations of cardiac cells[52]. Mixed populations of cells derived from human cardiac biopsies (cardiospheres) that are c-Kit+ Sca-1+ Flk1+ can self-renew and differentiate into cardiomyocytes by co-culture with postnatal rat cardiomyocytes[53]. These cardiospheres can be obtained in sufficient cell numbers for transplantations.

The postnatal cardiac progenitor marked by Isl1+ can self-renew *in vivo* and *in vitro*[54] and differentiate into functionally mature cardiomyocytes by co-culture with neonatal cardiomyocytes[48]. Moreover, Isl1+ marks the embryonic multipotent SHF progenitors and its derivatives[54, 55].

1.4. The heart

The embryonic heart is the first organ to function during development, and is comprised of diverse muscle and non-muscle cell lineages: atrial and ventricular cardiomyocytes, endocardial cells, vascular components, connective tissues, conduction system, smooth muscle and endothelial cells of the coronary vasculature[56].

1.4.1. The origin of the heart

The mouse embryo develops from the fertilization to the blastocyst structure prior to implantation, between embryonic day (E) 3.5-4.5. The blastocyst is a spherical structure that contains two distinct cell types at E3.5 –the inner cell mass (ICM) and trophectoderm (TE). The segregation of the ICM (few hours later) give rise to the epiblast or the primitive endonderm (PE) –the outer most cells that overlies the ICM–[57].

Successive steps follow as a cell specification proceeds, and implantation takes place by E5.5. The TE mediates implantation and then expands to form the extraembryonic ectoderm (ExE). The PE gives rise to the parietal endoderm and the visceral endoderm (VE). And the epiblast originates both the somatic tissues and the germ cell lineages[57, 58].

After implantation, a cavity forms in the center of the epiblast and all the structures (conceptus) elongates along the proximal-distal axis. The differences in marker gene expression can be seen along this axis[57, 58].

Around E6.0, the formation of the primitive streak is the morphological indication if the onset of gastrulation, which results in the formation of the primary germ layers of the embryo –the ectoderm, mesoderm and endoderm–[58].

During gastrulation, myocardial progenitor cells derived from the epiblast, ingress into the anterior part of the primitive streak (PS) and start their migration in an anterior lateral region relative to the streak, where they organize as part of the lateral plate mesoderm (LPM) at E6.5. The LPM splits into two layers, the splanchnic (inner) and somatic (outer) mesoderm, and gives rise to the coleomic cavity between the two layers at E7.5. Myocardial progenitors are restricted to the splanchnic mesoderm. The myocardial progenitor cells migrate bilaterally and anteriorly on a semicircular trajectory, forming the cardiac crescent (CC), between and E7.5[59]. The CC is constituted by two cardiac progenitor populations, known as the first (FHF) and second (SHF) heart fields.

At E8, the CC fuses at the midline and gives rise to the FHF-derived heart tube, which emerges as a linear tube that starts looping immediately and becomes a looped heart at E8.5 [59].

The linear tube expansion is mediated trough endogenous cell proliferation and the recruitment of additional cells. The cells that are added to the linear heart tube have a SHF origin and they will contribute to the arterial and venous poles of the linear heart tube.

The septation of the fetal heart into a four-chambered heart is mediated by interactions of cardiomyocytes with epicardial, edocardial cells and cardiac neural crest (CNC) cells.

By day E10.5, the developing heart shows well-defined chambers connected to the pulmonary trunk and aorta at E14.5.

1.4.2. Cardiac progenitor contributions to the heart

There are three spatially and temporally distinct sources of heart cell progenitors: cardiogenic mesoderm cells, proepicardium cells and cardiac neural crest (CNC) cells[56].

The cardiogenic mesoderm cells form the major proportion of the ventricular, atrial and OFT myocardium. These progenitors also contribute to the endocardium, the conduction system and the aortic and pulmonary cushions.

During the process of cardiac looping, progenitor cells from the proepicardial organ (PEO) migrate onto the heart to form the epicardium (the outer-most layer of the heart), cardiac fibroblast and cells of the coronary vasculature. Also, PEO cells also can give rise to cardiomyocytes in the walls of the cardiac chambers and in the ventricular septum[60].

The CNC cells comprise a non-cardiac cell type that contributes to the formation of aortic-pulmonary septum, ecto-mesenchyme and aortic smooth muscle cells. These progenitors are an important source of signals during heart development.

1.4.2.1. Cardiogenic mesoderm cells

Cardiac progenitor cells (CPC) segregate early in development, around the onset of gastrulation (E5). The cell segregation results in a medial-lateral repositioning of the CPCs where the cardiogenic mesoderm is located, which later goes throught morphological changes driven by endodermal provided clues. The new localization is the so-called splanchnic mesoderm. As the cardiac progenitors continue to migrate anterior- and laterally relative to the PS, they form the CC, from the two-myocardial fields derives, the FHF and SHF. The FHF is the restricted source of the early left ventricle cells, whereas the SHF is the source of outflow tract (OFT) myocardium. Both lineages contribute to other parts of the heart.

In the mouse embryo, the early heart tube derived mainly from FHF progenitors and it has a left ventricle cellular identity. The heart tube expansion and the elongating OFT depend on cell contributions from the SHF, by adding cells surrounding the aortic sac, immediately anterior to the forming heart tube[61, 62].

The FHF progenitors are located more anteriorly and lateraly in respect to the SHF progenitors. This localization exposed initially the FHF to BMP and FGF family

members, as well as inhibitors of the Wnt pathway, resulting in the onset of cardiac differentiation[61].

The onset of cardiac differentiation is marked by the expression of key transcription factors that regulates cardiac lineages specification, such as Nkx2.5, Gata4 and Tbx5.

Although many efforts were made in heart developmental analysis in diverse animal models, no genes exclusively expressed in early FHF progenitors has been found yet, but SHF progenitors are marked by transcription factor Isl1[55]. Different signaling pathways control the fate of the SHF progenitors, FGF promotes progenitor cell proliferation, Shh-mediated signals from the endoderm and canonical Wnt from the neural tube are important for the proliferation and inhibition of cell differentiation. BMP, Notch and non-canonical Wnt secreted from the lateral plate mesoderm, promote the differentiation of SHF progenitors[62]. Isl1 expression in the SHF is regulated by canonical Wnt signaling[63], where the activation is necessary for proliferation, pre-specification, renewal and differentiation of the SHF progenitors[64].

1.4.2.2. Proepicardium organ

The proepicardium organ (PEO) is a small and transient organ that arises from the coleomic mesenchyme of the septum transversum at E8.5. During the process of cardiac looping (between at E9.5-E11.5) freely floating PEO cell vesicles are released from the PEO, which flatten and spread out on contact to the unsheathed myocardium, forming the outer-most layer of the heart called epicardium, cardiac fibroblast and cells of the coronary vasculature[65].

PEO progenitors markers so far described are the T-box transcription factor (Tbx18), transcription factors Wilms tumor 1 (Wt1)[60, 66], Sema3D, Scleraxis[67], as well as Nkx2.5 and Isl1 which are also respectively markers of the FHF and SHF[68].

PEO induction, growth and maintenance depend on opposing interactions between FGF and BMP signaling pathways. While FGF2 induces proepicardial fate in the posterior splanchnic mesoderm, BMP-2 drives myocardial differentiation. BMP-2 and FGF2-mediated effects involve Smad transcription factors and the activation of mitogen-activated protein kinase kinase 1/2 (MEK1/2). The BMP signaling via Smad drives

differentiation towards the myocardial lineage, which is inhibited by FGF signaling via MEK1/2 activation[69].

Once the epithelial cell sheet of the epicardium is formed, epicardial cells provide soluble factors that stimulate coronary vessel development as well as cardiomyocyte proliferation and differentiation. The induction of EMT in epicardial cells leads to epicardium-derived cells. The subsequent migration and differentiation of epicardium-derived cells can acquire cardiac fibroblast, vascular smooth muscle, coronary endothelial[70] and cardiomyocytes in the walls of the cardiac chambers and in the ventricular septum[60]. The signaling molecules that govern epicardial differentiation as well as compact zone growth include FGFs, Notch and RA.

1.4.2.3. Cardiac neural crest progenitors

The cardiac neural crest cells are a subpopulation of neural crest (NC) cells, originated from the dorsal neural tube between the otic placode to the posterior border of somite three, that undergo EMT and migrate towards the heart via the third, fourth and sixth pharyngeal arches. The pharyngeal arch arteries form as a bilaterally symmetrical series of arteries that connect the aortic sac to the paired dorsal aortas. Later in development, aortic arch arteries 3-6 remode into asymmetric great arteries which are the carotid, definitive aortic arch and the ductus arteriosus. CNC from the arches also migrate through the anterior SHF into the OFT where they form the endocardial cushions[56, 71-73]. A mosaic of regulatory signals control the migration of CNC cells including the signaling pathways: FGF-8, BMPs, TGF-β, Notch-Jagged, Wnt/β-catenin[74] and RA.

The subsequent morphogenesis and differentiation of CNC cells are strongly influenced by cell-cell and cell-matrix adhesion molecules, like NCAM and connexin43, endothelins, semaphorins, neurotrophins and VEGF. N-Cadherin modulates CNC cell motility, as well as CNC cell survival in the OTF. Also, micronutrients such as folic acid and vitamin A can influence the development of NC-derived structures [56, 72]. Several proteins can act as a transcriptional regulators of CNC cells including Ap2[75] and Cited2[76], HIRA among others. As a result of the imbalance of signaling pathways in CNC, congenital heart disease might appear, such as DiGeorge and velocardiofacial syndromes. The syndromes are characterized by interrupted aortic arch type B,

abnormal myocardial functions, OFT malformations (persistent truncus arteriosus, tetralogy of Fallot, DORV, hyplastic thymus, hypoparathyroidism) or abnormal origin of a right subclavian artery, ventricular septal defect, psychosis, and cleft lip and cleft palate in the case of velocardiofacial syndrome[71-73].

Despite the lack of existence of CNC-restricted lineage markers; there are gene markers for NC populations allowing to follow the fate of CNC cells, like P0, the wingless-type MMTV integration site family member 1 (Wnt1), PlexinA and transcription factor paired box gene 3 (Pax3)[71-73].

1.5. *CITED family*

The CITED (CBP/p300-interacting trans-activator with glutamic/aspartate rich carboxy-terminal domain) gene family consists in four protein members: the Cited1 (MSG1), mouse melanocyte-specific gene[77, 78]; the Cited2 (MRG1), human MSG1-related gene 1/p35srj, serine-glycine rich junction protein[77-81]; the Cited3[82] and Cited4[83]. The members of this family function as transcriptional co-activators with non DNA-bindings domains family.

As the family name denotes, these factors interact with the histone acetyltransferases (HAT) homologous proteins CBP (cAMP-response-element binding protein) and p300 CBP/p300, that are co-activators linking upstream transcription factors to the basal transcriptional machinery, and also can acetylate the N-terminal tails of histones H3 and H4 in the nucleosomes. CBP/p300 form with other acetyltransferases, a coactivator complex that is capable of multiple acetylation events and in this manner regulates transcriptional activation[84, 85].

The CITED interaction domain with CBP/p300 resides in the carboxy-terminus of the Cited proteins, the CR2-CITED domain, which has a high binding affinity of to the first cysteine-histidine-rich (CH1) region of CBP/p300[79]. Moreover, in cells CITED2 protein can be found in complex with CBP/p300, although a fraction of CBP/p300 is not in complex with Cited2[79].

CITED2 and CITED1 share homology in two conserved domains, the conserved region 1(CR1), of 14 aminoacid (aa) and the conserved region 2 (CR2), encompassing 49 aa (Figure 1.3). This region, CR2, presents 76% identity between the Cited family

members and is strictly conserved among human and mouse, and is essential for CBP/p300 interaction *in vivo*[79, 86].

1.5.1. CITED2

CITED2 (p35srj/MRG1) is a nuclear protein ubiquitously expressed in multiple human tissues, and the protein is expressed in many if not all human and murine cell lines[79]. Cited2 homologous are found in vertebrates, such as human[87], mouse, chicken[77], but not in invertebrates such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans* or *Drosophila*[79].

1.5.2. Cited2 gene

The human and mouse CITED2 have a conserved acidic domain (CR2) (94.8% identity)[79], that is functionally active and which serve as an activator subunits of a multi-subunit transcription factor[86]. The human CITED2 gene localized in the chromosome 6q23.3 encodes one protein with one spliced isoform which is more expressed and more ubiquitous, named p35srj (srj stands for serine-glycine rich junction) than the other variant MRG1 (human MSG1-related gene 1). CITED2 gene contains three exons, separated by two small introns. Both introns are spliced out in MRG1 and intron 1 is transcribed, whereas the open reading frame of the p35srj cDNA begins in exon 2 and continued until exon 3. The exon 2 encodes the srj domain, present in p35srj but absent in MRG1, although it retains the high-affinity CBP/p300-binding[79].

The p35srj is the major endogenous isoform[80, 82], and because all data herein after refer to the isoform p35srj, I will use the term Cited2.

Mutations in CITED2 gene are thought to be disease causing in humans. The mutations are associated with congenital heart diseases like cardiac septal defects as well as outflow tract abnormalities associated with malrotation of the great arteries. Many of the variants identified are clustered in the srj junction domain, which is a domain present only in Cited2 and not the other Cited-family members (Figure 1.3). These CITED2 mutations (S170-G178del, G178-S179ins9, S198-G199del, T166N, R92G, N62S, A187T, G180-A187del, G184S, S192G, S192fs) alter the amino acid sequence and cluster in the SRJ domain of the protein[87-89]. However, none of the CITED2 mutations identified were shown to alter the cellular localization or expression levels of the

protein which is normally expressed the in nucleus[87].

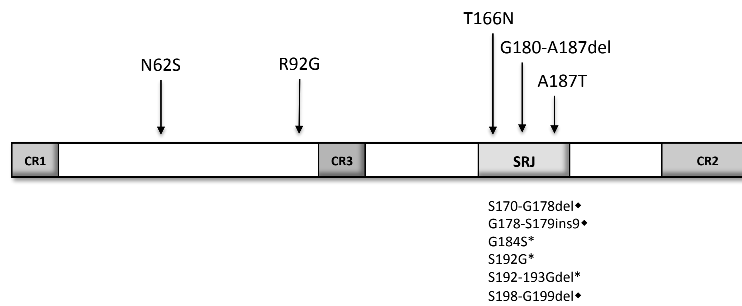


Figure 1.3. CITED2 mutations identified in human patiens with cardiac malformations.
Schematic diagram of human CITED2 showing conserved regions 1-3 (CR1-3) and the serine-glycine rich junction (SRJ). The location of CITED2 variants are indicated[88]. Variants found in Caucasian(◆)[87] and Chinese(*)[89] patients with CHD, are shown below the diagram.

1.5.3. Cited2 expression and protein function

CITED2 expression is induced by many factors including cytokines, insulin, serum, lipopolysaccharide, shear stress and hypoxia in diverse cell types[79, 80, 90].

The CITED2 promoter sequence has four putative hypoxia responsive elements (HREs, 5'-RCGTG) arranged in tandem within CpG islands. Also, it contains one consensus NFkB binding site upstream of the CITED2 promoter and 22 putative STAT binding sites[81]. Hypoxia inducible factor 1-α (HIF-1α) and CITED2 both bind directly to the CH1 region, under hypoxic condition, the activator of HIF-1α induces the synthesis of CITED2/mRNA and protein, resulting in elevated levels of CITED2 that inhibits the transcriptional activity of HIF-1α , through competition for overlapping binding sites on CBP/p300 *in vitro* and *in vivo*[79, 91].

CITED2 act as a co-activator of transcription when is associated with nuclear receptors such as peroxisome proliferator-activated-receptor α subtype (PPARα) to enhance transcription of target genes like angiopoietin-like protein 4, forkhead c2, HIF-1α, MAPK-phosphatase 1. Among the subtypes of peroxisome proliferator activated receptors, the PPARα has a role in carcinogenesis[92].

Under mechanical stress conditions and in a transforming growth factor-β depending pathway, CITED2 is capable of displacing Est-1 from CBP/p300 and modulate matrix metalloproteinase (MMP) -1 and MMP-13 expression at both at the mRNA and protein levels[90].

Moreover, CITED2 directly interacts *in vivo* with the LIM domain of Lhx2 and Lhx3 (LIM homeodomain factor-2 and -3), forming a complex and activate glycoprotein hormone α-subunit gene expression[93].

All the three TFAP2 isoforms interact with CITED2 via its c-terminus CR2 region which also binds the CH1 domain of p300/CBP (Tfap2 is involved in neural crest and neural tube development) and it is a co-co-activator for TFAP2 *in vitro*[76]. In addition, TFAP2 isoforms and CITED2 synergistically activate the Pitx2c promoter and control Pitx2c expression in mouse embryonic hearts (Pitx2 isoform involved in cardiac development)[94]. The functional interaction between CITED2 and TFAP2 might also been relevant for normal heart development in humans, since it was recently reported that a deletion of human CITED2 gene resulting in a mutant CITED2 protein (S170 to G178) with impairing ability to co-activate TFAP2C, and this CITED2 deletion is associated with congenital heart defect phenotypes in the patients[89 87]. Interestingly, another CITED family member, Cited4 has been shown to co-activate TFAP2 family members and to inhibit HIF1-mediated transcriptional activity[83, 95]. However, to date Cited4 has not been shown to play any critical role in mouse embryonic heart development[96], but it was recently reported that Cited4 is important for the proliferation of adult cardiomyocytes in healthy exercising adult mice[97].

The TGF-β downregulates Cited2/mRNA and protein expression in a dose depended manner, via Smad-2 and Smad-3, but not via Smad4 [98]. The regulation of Cited2 mRNA mediated by TGF-β is post-transcriptional, trough changes in the mRNA stability where its c-terminus is essential for the downregulation [98].

The CBP/p300 ability to be recruited to specific DNA sequence is crucial for Smad-mediated transcription, in which the transcriptional activity resides in the MH2-domain of Smad proteins [99]. The c-terminal region of CITED2 is essential for the interaction with MH2 domain of Smad3, and p300 enhances this interaction; this complex is involved in the transcription control of the MMP-9 gene[100].

1.5.4. CITED2 expression in mouse development

As previously described, CITED2 regulates gene transcription by interaction with other transcription factors acting as a co-activator or co-repressor[79].

Cited2 is expressed in mESC[77], and its overexpression sustains self-renewal of mESCs, even in the absence of LIF[101, 102]. Moreover, Cited2 is a direct target of FoxP1 which mediates pluripotency in mESC[103], and Cited is involved in the maintenance of foetal and adult hematopoietic stem cells[104, 105]. The absence of Cited2 during ESC differentiation leads to a delay in self-renewal genes affecting hematopoietic, neuronal and cardiac differentiation. Moreover, and has been proposed that defective cardiomyocyte differentiation is caused by a reduction in the expression of NFAT3 and its target genes, leading to a defective cardiomyocyte differentiation[106].

Furthermore, CITED2 expression is widespread during mouse development[77], and at the pre-gastrulation stage, E6.5 Cited2 expression accumulates in the anterior visceral endoderm (AVE), in the forming primitive streak (anterior endoderm). Its starting expression is first visualized in the posterior embryonic endoderm and in the nascent mesoderm. Later, Cited2 expression accumulates in the ventral node (caudal lip and in the depression of the node), and is also detected in blood islands (E7.5) and by E8 the expression is detected in the cardiac crescent, anterior lateral mesoderm and in trunk paraxial mesoderm. Cited2 expression at E9.25 is widespread in the heart, aortic sac, in the atria and sinus venous and in the myocardium adjacent to the endocardial cushions of the outflow tract (OFT) and the atrioventricular (AV) canal. Cited2 is also is present in the dorsal neuroectoderm at the forebrain-midbrain boundary, and in the hindbrain. Its expression is also detected in the branchial arches 1 to 3 and in the somites. And by E10.5, Cited2 transcript are detected at the branchial arch 4, persist in the OFT and the AV canal. At E13.5, the expression is restricted to the outflow and inflow regions of the heart, AV cushion, septum primun and the tip of the muscular ventricular septum, during this stage, Cited2 is found predominantly in the myocardium, also in endocardial cells, endocardial cushions. Later, Cited2 is ubiquitously expressed in adult adult tissues[79].

More importantly, Cited2 has been shown to be important for mice development and loss of Cited2 affects trophoblast formation and vascularization of the mouse placenta[107] and mice present perinatal lethality, displaying a numerous developmental defects in organs and tissues heart, lung, liver and eyes[76, 108, 109]. Moreover, in Cited2-null mice with heart defects (heterotaxia, which is associated with cardiac malformations)[76], around 80% of Cited2-null embryos present exencephaly, anterior open or incomplete close of the neural tube[76, 110], and loss of adrenal glands. The

exencephaly and defects in neural tube phenotype are a consequence of the imbalance in cell proliferation and cell survival in the neuroephitelium (forebrain-midbrain boundary) and/or hindbrain, and the phenotype can be rescued by exogenous administration of folic acid *in vivo* and *in vitro*[110]. Among heart defects Cited2-null mice present a broad spectrum of cardiac defects including double outlet right ventricle, atrial septal defect, ventricular septal defect, overriding aorta, persistent truncus arteriosus, and pulmonary artery stenosis[76, 91, 110].

Cited2 is required for the establishment of the left-right axis in mouse development [111] by controlling the Nodal-Pitx2c pathway[94].

Chapter II

Objectives

2. Objectives

Cited2 knockout from the epiblast in results in the death *in utero of* mice embryos, which exhibit a mosaic of developmental defects, amongst which cardiac defects and lack of adrenal glands were always present. The most common heart defects identified in Cited2-knockout embryos were t ventricular septal defects. Interestingly, Cited2-knockout embryos also displayed alterations in the left-right asymmetric axis patterning resulting in the impairment of the transcriptional activation of Pitx2c promoter by TAFP2 protein which require Cited2 to enhance their transcriptional activity[94]. Pitx2c isoform is the responsible for the proper left-right asymmetry of the heart, as well as other organs. These observations suggested an important role played by Cited2 in heart development in mice and led to the several studies that also pointed out the association of variations in the genomic sequence of human CITED2 and congenital heart defects in human patients.

In an effort to better define the spatio-temporal requirement of Cited2 function in heart development, conditional knockouts of Cited2 have been carried out in the early mesoderm, cardiac mesoderm and in neural crest cells[112]. Curiously, none of the conditional Cited2-knockouts recapitulated the dramatic cardiac phenotypes observed when Cited2 is knocked out as early as the epiblast stage. Therefore, the cell types and tissue-expression requiring the expression of Cited2 for the correct cardiac development to proceed, as well as the molecular mechanisms that involve Cited2 for this process remain largely unknown. Moreover, a likely hypothesis that can be drawn from the conditional Cited2-knockouts published is that Cited2 might play a marked role in the molecular events occurring between the epiblastic stage and the mesodermal tissue differentiation.

Since ES cell differentiation *in vitro* recapitulates early steps of embryonic development, we have used this cellular system to study the role of Cited2 in ES differentiation towards cardiac cell lineages, and get insights into Cited2 functions in the early embryonic development. Moreover, pluripotent stem cells are considered as a potential source for cells that would be used for many and diverse future therapies. Thus, understanding the role of Cited2 in cardiac differentiation from ES cells would

also shed light in the molecular mechanisms controlling cardiac cell fate that might be useful to reliably establish pluripotent-derived cardiac cells for future therapies.

Overall, the main objectives of the present work are:

1. To determine whether Cited2 is critical for ES cell differentiation to cardiac cell lineages;
2. To characterize the time points and cell types where Cited2 function is critical for cardiac differentiation;
3. To identify Cited2-target genes involved in the cardiogenic differentiation process;
4. To determine the impact of CITED2 overexpression on the commitment of mES cells;
5. To confirm and to study the physical and functional interactions between Cited2 and pro-cardiogenic transcription factors.

The completion of this work will reveal important mechanisms to understand Cited2 cellular functions in cardiac cell lineages differentiation processes from ES cells.

Chapter III

Methods

3. Methods

3.1. Cell culture

3.1.1. Embryonic stem cell lines

All ESC lines were cultured on 0.1% (SIGMA) gelatin-coated plates in undifferentiating medium supplemented with LIF[101] and cultured at 37°C with 5% CO_2. The cultures were monitored under inverted microscope.

The Cited2$^{flox/flox}$ ESC line harboring Cited2 alleles flanked by LoxP sites were isolated from blastocysts of mice described elsewhere[112]. Cited2$^{flox/flox}$ were stably transfected with the pPyCAGIP-CreERt plasmid (encoding Cre-ERT2[113] that we subcloned into pPyCAGIP vector). Three independent clones were obtained, we used the C2$^{fl/fl}$[Cre]B hereafter referred as Cited2$^{fl/fl}$. Knockout *Cited2* were obtained upon treatment with 1μM of 4-hydroxy-Tamoxifen (4HT) during the indicated times.

Mouse embryonic stem cells E14/T were a gift from Professor Austin Smith (University of Cambridge, UK) described elsewhere[114]. E14/T cells were stably transfected with the pPyCAGIP (empty, control) or pPyCAGIP-FlagCITED2 (encoding human CITED2 protein tagged with flag peptide at its N-terminal domain), that are able to replicate in an episomal form in E14/T cells and harbor the puromycin resistant gene allowing their selection in culture (2μg/ml puromycin).

The AD2 Apple mESC line were a gift from Ibrahim Domian (Cardiovascular Research center, Massachusetts General Hospital, USA) and were previously described[115]. The AD2 cell line harbors the GFP gene under the control of the *Nkx2.5* promoter and the dsRed gene under the control of second heart field enhancer regulatory elements. In pluripotent state, cells neither expressed GFP nor dsRed fluorescence proteins. The fluorescence starts to appear by day 2 during differentiation by EB cultures.

Undifferentiated medium for E14/T, Cited2$^{fl/fl}$ and cell lines derived from those cells was composed as follows: GMEM 1X (SIGMA, G5154); 4mM L-Glutamine (200mM, GIBCO® Invitrogen™); 1mM of sodium pyruvate (GIBCO® Invitrogen™); 1x Non-essential aminoacids (GIBCO® Invitrogen™); 10% (v/v) Fetal bovine serum (FBS) (SIGMA); β-mercaptoethanol (GIBCO® Invitrogen™) to a final concentration of 0.05mM; 1/1000 penicillin/streptomycin mix (GIBCO® Invitrogen™) and 1000 Units/mL of LIF.

For the culture of AD2 cell line, we used KO-DMEM 1X (GIBCO® Invitrogen™); 2 mM L-Glutamine (200mM, GIBCO® Invitrogen™); 1x Non-essential aminoacids (GIBCO® Invitrogen™); 15% (v/v) FBS (HyClone); 1/1000 of β-mercaptoethanol (GIBCO® Invitrogen™); 1/1000 penicillin/streptomycin mix (GIBCO® Invitrogen™) and 1000 Units/mL of LIF.

Secondary heart field progenitors isolated from differentiating AD2 cells were maintained on a layer of mitotically inactivated mouse embryonic fibroblast. The undifferentiated culture media used was the same used for AD2 cell line, supplemented with BIO, ((2'Z, 3'E)-6-Bromoindirubin-3-oxime; SIGMA) to a final concentration of 2.5μM.

To induce ESC differentiation we used a hanging-drop method. The ESC differentiating medium was prepared with undifferentiated-medium GMEM containing 20% FBS and without LIF for E14/T-derivatives as well as for Cited2$^{fl/fl}$ cell line. For the AD2 cell line undifferentiated-medium KO-DMEM and 20% FBS (Hyclone) without LIF, was used.

Briefly, 500 (E14/T and AD2) or 1000 (Cited2$^{fl/fl}$) cells were cultured in 20μL of their respective ESC differentiation medium and were spotted on a bacterial petri dish lid, at 37°C with 5% CO_2. After 48 hours the EBs formed were transferred to a suspension culture, and supplemented with 10mL of ESC differentiation medium. By day 5, the EBs were transferred to 0.1% gelatin coated 60-cm tissue culture dishes to allow cell to attach. To assess the progression of differentiated cultures, we used an inverted microscope, and samples were obtained at different time points during differentiation.

3.1.2. In vitro cell cultures

Human embryonic kidney cell line, HEK-293T, (293T, ATCC® CRL-3216™) were maintained in tissue-cultured dishes with DMEM medium (SIGMA); 2mM L-Glutamine (200mM, GIBCO® Invitrogen™); 1/1000 penicillin/streptomycin mix (GIBCO® Invitrogen™) and 10% FBS heat inactivated (SIGMA).

3.1.3. Transfections and plasmid vectors

For DNA transfections, independently of the ESC line used, the cells were plated in gelatin-coated 6-well plates at $2.5x10^5$ cells per well, 3μg of total DNA vectors were transfected with Lipofectamine 2000 (Invitrogen). After 5 hours ESC medium and LIF were added to cultures, and one day after transfection antibiotic were added to cultures.

For Bi-molecular fluorescence complementation (BiFC), E14/T cells were plated in gelatin-coated 6-well plates at $2x10^4$ cells per well, transfected with 250*ng* of individual vectors. Fluorescence detection of transfected cells will be originated by the close proximity of the residues 1–155 and residues 156–239 of Venus-YFP. Cells harboring the plasmids were selected for their resistance to puromycin and for the emission of fluorescence.

pPyCAGIP vector (with a puromycin resistant gene) was provided by Austin Smith (University of Cambridge, UK) and described elsewhere and its derivative FC2 (pPyCAGIP-FlagCITED2) vector that encodes human CITED2 protein tagged with flag peptide at its N-terminal domain[101, 102, 114]. The vectors used for Cited2 knockdown and the control knockdown were previously prepared in the group. Briefly, this plasmid was constructed by insertion of a 375bp cDNA fragment corresponding to the amino acids 2 to 123 of the human CITED2 at the BamHI of the pDoubNeo vector [116]. This vector produces a double stranded RNA targeting Cited2 messenger. The Control vector was constructed by insertion of a 369bp fragment of the Pol region containing a splice acceptor site of the Moloney mouse leukemia virus at the BamHI of the pDoubNeo

vector. The control vector produces a double stranded RNA that has no target in normal mouse cells.

For BiFC assays, the pPyCAGIP vector was modified to obtain the following vectors:

- VEC-CAGIP, vector expressing the *C*-terminal fragment (residues 156–239) of Venus-YFP (VEC).
- VEN-CAGIP, vector expressing the *N*-terminal fragment (residues 1–155) of Venus-YFP (VEN).
- VEC-Cited2, vector expressing the *C*-terminal fragment (residues 156–239) of Venus-YFP fused in frame to a flag-tagged CITED2 at its *N*-terminal region.
- VEN-Isl1, vector expressing the *N*-terminal fragment (residues 1–155) of Venus-YFP fused in frame to a myc-tagged ISLET1.
- VEN-CH1, vector expressing the *N*-terminal fragment (residues 1–155) of Venus-YFP fused in frame to the CH1 domain of p300, which is the domain of p300 interacting with CITED2 with high affinity[79].

3.1.4. Ribonucleic acid isolation and real-time polymerase chain reaction

Total ribonucleic acid (RNA) was extracted using a Trizol based method, TRI-Reagent (Sigma) or purified by Direct-zol™ RNA MiniPrep (Zymo Research). 0.5 μg of total RNA were used for reverse-transcription according to the manufacter's protocol, using either qScript cDNA supermix (Quanta Biosciences) or Superscript II reverse transcriptase (Invitrogen). Semi-quantitative real-time polymerase chain reaction (qRT-PCR) was performed using either PerfeCTa SYBR Green Fast Mix (Quanta Biosciences) or SsoFast™ EvaGreen® supermix (BioRad), in a CFX96™ Real-Time PCR detection system (BioRad), and using the CFX Manager™ Software (BioRad).

The list of primers used for qRT-PCR is presented in the Table 3.1.

3.1.5. Western blot and Immunoprecipitation of proteins

Protein lysates were prepared in cold lysis buffer (Tris 50 mM (pH7.5), 180mM NaCl, 0.5% NP40) supplemented with complete protease inhibitors (Roche), 1mM DTT, 100µM PMSF (Phenylmethanesulfonyl fluoride, SIGMA), 0.2µM of sodium orthovanadate (Na_3VO_4, SIGMA). Final protein concentrations were measured using Bradford protein assay kit (SIGMA). Approximately 20µg of protein lysates were separated by SDS-PAGE on a 10% bisacrylamide-polyacrylamide (29:1) gel using the Miniprotean II system (BioRad) along with the NZYColour Protein Marker II (NZYTech), and transferred to PVDF membrane (GE Healthcare life sciences) using a Semi-dry blotter (30 min at 200mA, BioRad). The PVDF membrane was blocked for approximately one hour at room temperature with a solution containing 6% milk diluted in PBS with 0.1% Tween20 (PBST). The membrane was subsequently incubated with the indicated primary antibody at a working dilution of 1/2000 (diluted in 6% milk-PBST), except otherwise mentioned, at room temperature environment (RT). Additional incubation for 1 hour at RT with the adequate secondary antibody at a working dilution of 1/5000 in 6% milk diluted in PBST. The horseradish peroxidase (HRP) activity coupled to the secondary antibody was revealed using the ECL Plus kit (GE Healthcare life sciences) according to manufacturer's instructions, and light by the reaction was detected by autoradiography (GE Healthcare life sciences) or by image acquisition on ChemiDoc™ MP Imaging System (BioRad).

For immunoprecipitation, $1x10^6$ E14/T cells were grown in conditions maintaining the pluripotent state, protein lysates were prepared as described above, except that DTT was omitted in the buffer when agarose beads coupled to anti-Flag M2 (AFM2) were used (Sigma). $1/10^{th}$ of the total protein extract was kept and used as a input reference in the SDS-PAGE gels. Protein G Sepharose beads (GE Healthcare life sciences) pre-blocked with 5% bovine serum albumin (BSA, Sigma) were used for pre-clearing the extracts during 30 min. The primary antibody or the anti-Flag M2 antibody coupled to sepharose beads (40µL/sample) were added to the pre-cleared lysates and incubated overnight at 4°C. Primary anti-myc antibody (Abcam) was used at a dilution of 1:250, and anti-Isl1 (SantaCruz) at a dilution 1:100 for the immunoprecipation step.

The totality of the protein extracts immunoprecipitated with AFM2 beads was separated using a SDS-PAGE 10% gel on a Emperor Penguin™ Electrophoresis System

(Owl) and transferred to a PVDF membrane (GE Healthcare life sciences) as described above. Blots were probed with the indicated primary antibody and subsequently incubated with the corresponding secondary antibody–HRP conjugated as described above for western blotting proceedings.

For the detection and immunoprecipitation of human and mouse Cited2 protein, we have used the mouse monoclonal (JA22) anti-Cited2 (ab5155, Abcam), for the detection of flag-tagged proteins we have used anti-FLAG® M2 (SIGMA), and for immunoprecipitation of flag-tagged proteins, we have used the anti-FLAG® M2 Affinitty Gel (A2220, SIGMA), the mouse monoclonal anti-Gata4 (sc-25310, Santa Cruz) was used to detect mouse Gata4 protein. Rabbit anti-Isl1 polyclonal antibody (EP4182, Santa Cruz) was used to detect and to immunoprecipitate Isl1 peptide. A rabbit polyclonal antibody specifically recognizing Myc tag (Abcam) was used to detect and immunoprecipitate myc-tagged peptides. A mouse monoclonal antibody anti-β-Tubulin (T5293, SIGMA) was used to assess the equal loading of the samples in SDS-PAGE.

3.1.6. Chromatin immunoprecipitation assay

$1x10^6$ E14/T cells were grown in conditions maintaining the pluripotent state of the cells and fixed with 1% formaldehyde (SIGMA) and quenched by glycine to final concentration of 0.125M (SIGMA), before the chromatin break down by sonication (500-1000 base pairs via Ultrasonic processor, SONICS, Vibra cell™, 4 × 30 seconds, with 1 minute, amplitude 50). The chromatin-protein complexes were immunoprecipitated with 22.4μg/μL of mouse monoclonal anti-flag antibody (Sigma) or 3 μg/μL of a control mouse monoclonal antibody anti-cytochrome C (Abcam). The immunoprecipitated DNA was purified by phenol-chloroform method, and used as a template in real time RT-PCR using primers specifically amplifying elements of the mouse Isl1 locus and fragments of the Nkx2.5, Gata4 and Tbx5 promoters (primers sequences described in Table 3.1). We quantified by qRT-PCR the enrichment of each genomic regulatory element compared to input DNA (chromatin purified from E14/T extracts without immunoprecipitation) and normalized to the amplification of Gapdh promoter amplification, and the results from two independent experiments were presented as a percent of the total input chromatin

3.1.7. Fluorescence-activated cell sorting

AD2 cell line were cultured under differentiated conditions at indicated time points (EBs containing 500 cell/drop). The EBs were dissociated into single cells (using trypsin 0.25%, GIBCO® Invitrogen™), centrifuged at 1000 rpm for 3 min, and suspended in 700µL of cold PBS 1x supplemented with 1% of FBS (Hyclone) and gentamycin (SIGMA) to final concentration of 5µM, and subjected to flow cytometry FACSAria II Cell Sorter (BD Bioscience). Based on the presence of GFP and dsRed fluorescence, four populations were isolated for RNA extraction.

For FACS analysis for GFP and dsRed fluorescence in AD2 cells were subjected to flow cytometry, in FACS Calibur (BD Bioscience).

3.1.8. Alkaline phosphatase and cell proliferation assays

Alkaline phosphatase assays and stem cell fraction (SF) were performed as previously described[101]. For cell proliferation assays of 1000 cells transfected with pDC2 or control vectors were plated in gelatin-coated 12-well plates and counted at the indicated times using a hemocytometer. For cell proliferation assays with Cited2-null and control, 10000 cells per well were plated in gelatin-coated 12-well plates and counted using a hemocytometer and plated at the same density every 3 days. Population doubling per passage and cumulative population doubling (CPD) at each passage was calculated as described previously [117].

Table 3.1. List of gene-specific primers and annealing temperatures for Real-Time PCR.

Gene	Forward 5'-3'	Reverse 5'-3'	(°C)*
α-MHC[118]	GATGGCACAGAAGATGCTGA	CTGCCCCTTGGTGACATACT	65
Activin[119]	CTCCACGATCATGTTCTGAAT	GATGATGTTTTGACCATCATC	60
AFP[120]	GCCACCGAGGAGGAAGTG	AGTCTTCTTGCGTGCCAGC	65
β-III-tubulin	TATTCAGGCCCGACAACTTT	GGGTGTCAACCAGAGGAAGT	60
Brachyury (T)[121]	CTCTAATGTCCTCCCTTGTTGCC	TGCAGATTGTCTTTGGCTACTTTG	65
Cited2	CGCATCATCACCAGCACCAG	GCGTTCGTGGCATTCATGTTG	65
Cx40[122]	CAGAGCCTGAAGAAGCCAAC	ATGCGGAAAATGAACAGGAC	60
CycD1[122]	TCCTGCTACCGCACAACGC	CCAGCTTCTTCCTCCACTTCCC	60
CycD2[122]	CTGGCCAAGATCACCCAC	CACGTCTGTAGGGGTGGTG	60
FoxA2[120]	GGCCCAGTCACGAACAAAGC	CCCAAAGTCTCCACTCAGCCTC	65
GAPDH	TCCCACTCTTCCACCTTCGATGC	GGGTCTGGGATGGAAATTGTCAGG	65
Gata4[121]	TTCCTGCTCGGACTTGGGAC	TTCCCAGGCAGGTGGAGAATAAG	60
Gata5[121]	AGACCTGGACACAGGCAACACC	AAAGGCAGAGAAACCAACGTGG	60
Gata6[121]	ACAGCCCACTTCTGTGTTCCC	GTGGGTTGGTCACGTGGTACAG	60
Gsc[121]	AAACGCCGAGAAGTGGAACAAG	AAGGCAGGGTGTGTGCAAGTAG	60
Hand1[121]	GCGTCAGTACCCTGATGCCTTC	AAAGAGGAGGTAAGAGGACGGAAG	65
Isl1[121]	CTTAAGCATGCCCTGTAGCTGG	CAGACAGGAGTCAAACACAATCCC	65
Mef2c[123]	AGGCACCAGCGCAGGGAATG	CCACCGGGGTAGCCAATGACT	65
Mesp1[123]	TGTACGCAGAAACAGCATCC	TTGTCCCCTCCACTCTTCAG	65
Nanog[124]	CTCATCAATGCCTGCAGTTTTTCA	CTCCTCAGGGCCCTTGTCAGC	65
Nestin[118]	GGTGCTGAGTATGTCGTGGA	CGGAGATGATGACCCTTTTG	60
Nkx2.5[118]	CCACTCTCTGCTACCCACCT	CCAGGTTCAGGATGTCTTTGA	65
Nodal[125]	TGGCGTACATGTTGAGCCTCT	TGAAAGTCCAGTTCTGTCCGG	65
Nppa[122]	CGGTGTCCAACACAGATCTG	TCTCTCAGAGGTGGGTTGAC	60
Oct3/4[121]	GCAGGAGCACGAGTGGAAAGCAAC	CCAGGCCTCGAAGCGACAGATG	65
Pitx2c[126]	CTTGGAGCACCGAGCAGC	CTGGAAAGTGGCTTCCAG	65
Sox17[121]	AAGAAACCCTAAACACAAACAGCG	TTTGTGGGAAGTGGGATCAAGAC	65
Sox2[121]	CGAGATAAACATGGCAATCAAATG	AACGTTTGCCTTAAACAAGACCAC	65
Tbx5[121]	GGACCCAGTCCCTTGAATGG	TCCAGGCTGAGGAGTTCTAGGC	65
Tnt[118]	GAGGAGGTGGTGGAGGAGTA	GGCTTCTTCATCAGGACCAA	65
Vegf[127]	GTCCCATGAAGTGATCAAGTTC	CCGCTCTGAACAAGGCTCAG	65
Vegfr2/Flk1[127]	ACTGCAGTGATTGCCATGTTCT	TCATTGGCCCGCTTAACG	60

* Annealing temperature

ChIP	Forward 5'-3'	Reverse 5'-3'	(°C)*
Nkx2.5 promoter[128]	AGGCAAAGAAATCACTCCACA	TGTTACAATGGCTGGGAAGG	60
Tbx5 promoter[128]	GAAGCATTTTCTATACTTTGTGAGCA	TCAGCCAGCTGTTTTCAGAG	60
Gata4 promoter[128]	ACTCCCTTAGGCCAGTCAGC	GGAAAAGAGCAGGGACTCG	60
Isl1 promoter[129]	TTTTGGGTCTAACCGTCCTACTC	CCGCTTTCCTTCACTGACTC	60
First exon of Isl1[129]	ACTATTTGCCACCTAGCCACAG	AGAGGGAGTAATGTCCACAGTG	60
Last exon of Isl1[129]	GGTAGCAACACTGTGAAGACAATC	GAATAGCATATGTTGGGCTTAGGG	60

* Annealing temperature

Chapter IV

Results

4. Results

4.1. Establishment and characterization of Cited2$^{flox/flox}$ stem cell line

We have showed by overexpression and knockdown approaches in mouse ESCs that Cited2 is involved in the proliferation and maintenance of pluripotency, at least in part by controlling Nanog expression (data not shown).

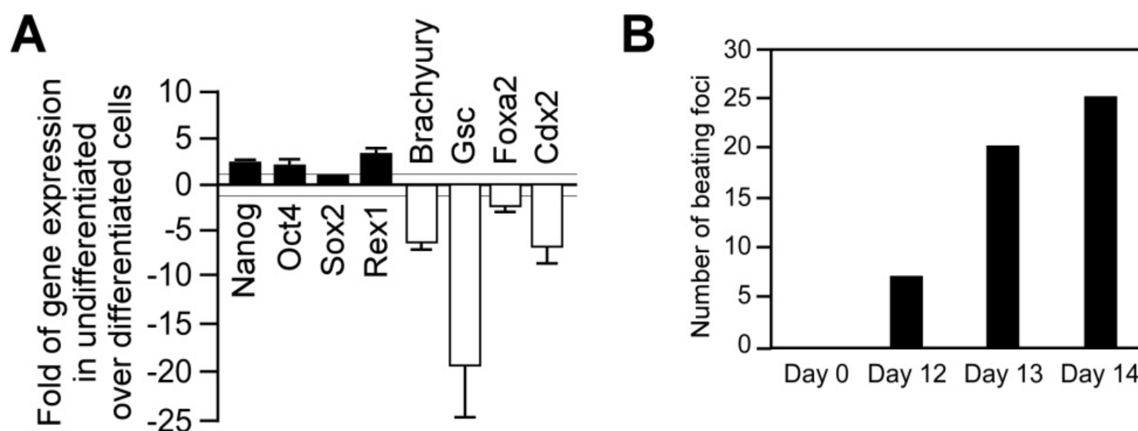


Figure 4.1. C2$^{fl/fl}$ ESC have pluripotent features.
(A) C2$^{fl/fl}$ ESCs were differentiated by hanging drop method and removal of LIF. Expression of pluripotency transcripts (black bars) and differentiation markers (white bars) were determined by qRT-PCR in C2$^{fl/fl}$ ESCs treated with 1μM 4HT or ethanol (vehicle for 4HT) for 48 hrs. Expression levels are normalized for Gapdh and reported as a fold of expression relative to C2$^{fl/fl}$ ESCs treated with ethanol. Results are presented as the mean ± s.e.m. of three independent biological replicates (each performed in technical duplicate). The panel shows that markers of pluripotency decreased while markers of differentiation are expressed. (B) The emergence of cardiac beating foci from C2$^{fl/fl}$ ESC-derived embryoid in culture is presented at the indicated days. Figure adapted from the manuscript Kranc et al. – Submitted to STEM CELL REPORTS.

To further demonstrate the role of Cited2 in ESCs, we have derived in collaboration with Dr. Kamil Kranc (University of Edinburgh) and Dr. Tariq Enver (University College of London), and characterized ESC lines harboring Cited2 floxed alleles (Cited2$^{fl/fl}$). For this purpose, mice Cited2$^{fl/fl}$ previously described were intercrossed, and at the blastocyst stage, ESCs were derived (hereafter called C2$^{fl/fl}$ ESCs) in which exon 2 of *Cited2* is flanked by LoxP sites. C2$^{fl/fl}$ ESC colonies were maintained in culture with LIF supplementation showed a typical ESC cell morphology, expressed pluripotency markers and alkaline phosphatase (AP), which also mark pluripotency. Moreover, the C2$^{fl/fl}$ ES cells differentiated upon removal of LIF and by

formation of embryoid bodies originated spontaneous beating foci suggesting that some underwent a cardiac cell fate, they displayed a decreased expression of pluripotent markers while the expression of differentiation marker genes was enhanced (Figure 4.1 and 4.2A). Overall, these observations confirmed that the novel C2$^{fl/fl}$ ESC line, when expressing Cited2, was indeed pluripotent, able to self-renew in the standard ESC cultivation conditions, and more importantly these cells conserved the ability to undergo a cardiac cell lineage differentiation.

4.2. CITED2 loss-of-function experiments in embryonic stem cells

The C2$^{fl/fl}$ ESCs have the alleles coding for Cited2 flanked by loxP, and the transient transfection of a vector expressing a constitutively active Cre recombinase in these cells was able to excise Cited2 alleles and activate the expression of the LacZ cassette present in C2$^{fl/fl}$ ESC has described in knockout embryos[112].

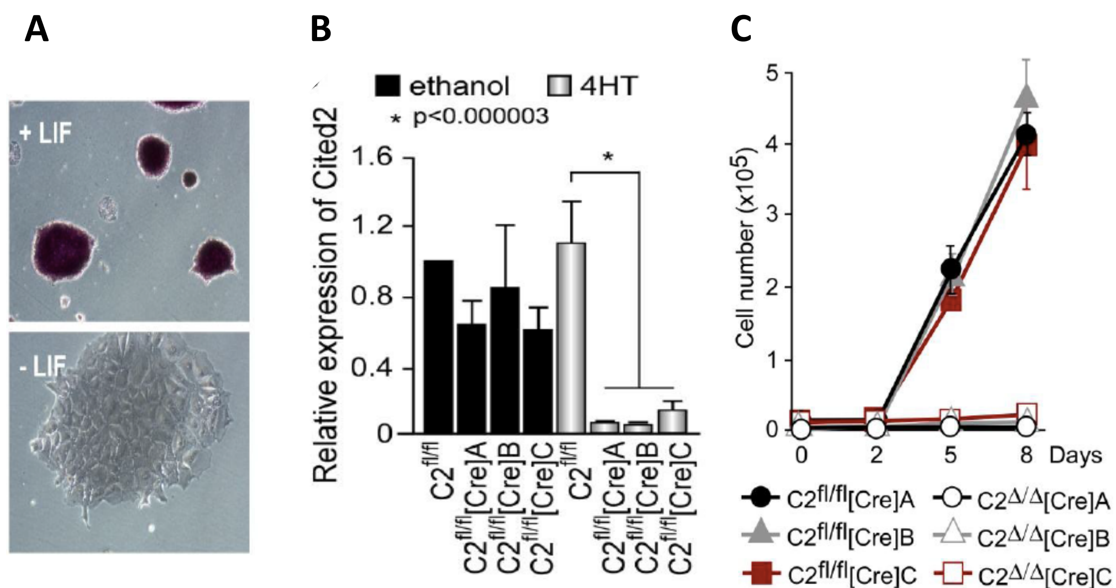


Figure 4.2. C2$^{fl/fl}$ ESC have pluripotent features and C2$^{fl/fl}$[Cre] ESCs clones presented indistinguishable behavior.

(A) Alkaline phosphatase (AP) activity in Cited2$^{fl/fl}$ ES cells cultured on gelatine in the presence (top panel) or 4 days after removal (bottom panel) of LIF. (B) Expression of Cited2 transcripts determined by qRT-PCR in C2$^{fl/fl}$, and C2$^{fl/fl}$ ESC stably transfected with a Cre-ERt expressing plasmid, the ESC lines C2$^{fl/fl}$[Cre]A, C2$^{fl/fl}$[Cre]B and C2$^{fl/fl}$[Cre]C, treated with 1μM 4HT or ethanol (vehicle for 4HT) for 48hrs. Expression level is normalized for *Gapdh* and reported as relative to the expression in C2$^{fl/fl}$ ESC treated with ethanol which is set at 1. Results are presented as the mean ± s.e.m. of three independent biological replicates (each performed in technical duplicate). (C) Proliferation of C2$^{fl/fl}$[Cre]A, C2$^{fl/fl}$[Cre]B and C2$^{fl/fl}$[Cre]C ESC lines plated at 500 cells per well of gelatinized wells (12-well plate) in the presence of ethanol or 0.5uM 4HT at day 0. Cells were maintained in culture with the conditions applied at day 0 until counted at the indicated time pionts. Results are presented as the mean ± s.e.m. of three biological replicates, each performed in technical duplicate.

In order to facilitate the excision of Cited2 alleles by the Cre recombinase, we generated novel mESC lines derived from the Cited2$^{fl/fl}$ ESC line which express the Cre recombinase in fusion to a mutated human estrogen receptor ligand-binding domain (Cre-ERt2). The Cre-ERt recombinase is constitutively expressed in an inactive form that can be activated by addition of tamoxifen or tamoxifen-derivatives to the culture medium. The novel ESC lines, hereafter named C2$^{fl/fl}$[Cre] ESCs, were obtained by stable transfection of a vector expressing the tamoxifen inducible Cre-ERt and puromycin selection for up to four weeks. Three independent clones of C2$^{fl/fl}$[Cre] ESCs were isolated and named C2$^{fl/fl}$[Cre]A, C2$^{fl/fl}$[Cre]B and C2$^{fl/fl}$[Cre]C. The clones presented indistinguishable behavior (Figure 4.2B-C). The activation of Cre-ERt by 4-hydroxytamoxifen (4HT) treatment significantly reduced endogenous Cited2 expression within 48 hrs in C2$^{fl/fl}$[Cre] ES cells while Cited2 expression in Cited2$^{fl/fl}$ ES cells was maintained at levels similar to those of vehicle (Ethanol) treated cells (Figure 4.2B-C). These observations indicated that the addition of 4HT to the culture medium results in the activation of the Cre recombinase and subsequent deletion of the *Cited2* alleles, giving rise to Cited2-depleted cells, hereafter referred as Cited2^{4HT} cells. Since the clones C2$^{fl/fl}$[Cre]A, C2$^{fl/fl}$[Cre]B and C2$^{fl/fl}$[Cre]C behaved so similarly, we decided to mainly use the clone C2$^{fl/fl}$[Cre]B for the rest of the work presented , some of the results were also confirmed using C2$^{fl/fl}$[Cre]A. The results presented here in this thesis, were obtained using C2$^{fl/fl}$[Cre]B-derived cultures, and here after will be refered as Cited2$^{flox/flox}$ for simplicity.

This inducible cell line allowed the deletion of Cited2 alleles at different time points as differentiation events progressed.

We confirmed the decrease of *Cited2* expression in mES Cited2^{4HT} cell cultures, by qRT-PCR and by western blotting analysis (Figures 4.3A and -B, respectively).

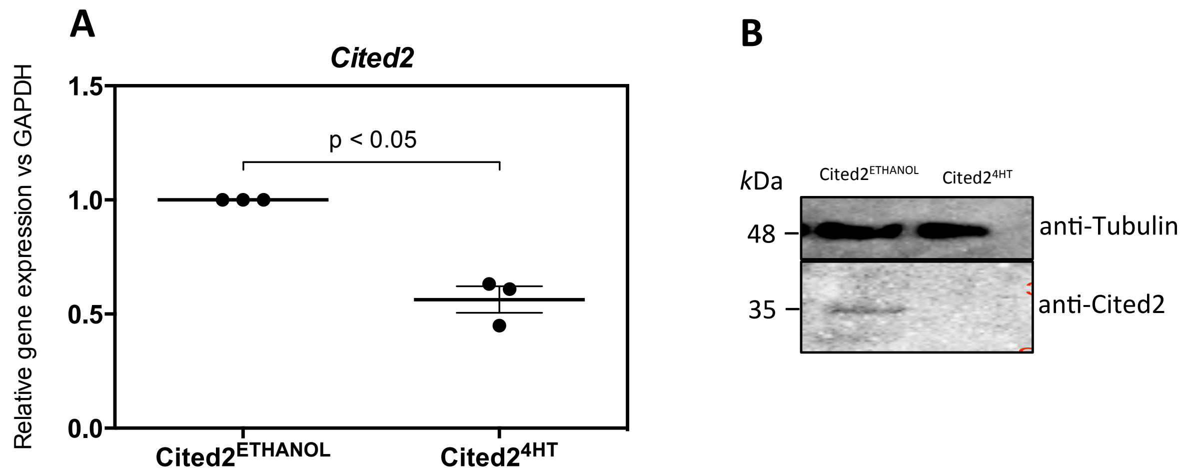


Figure 4.3. Gene expression and protein levels in pluripotent Cited2$^{flox/flox}$ cultures two days after treatment with vehicle or 4HT at day 0.

(A) Total RNA extraction from pluripotent Cited2$^{flox/flox}$ mES cultures obtained after two days of treatment with Tamoxifen (Cited2^{4HT}) or vehicle (Cited^{Ethanol}). The housekeeping gene glyceraldehydes 3-phosphate dehydrogenase (Gapdh) was used as a control. The levels of Cited2 expression in Cited2^{Ethanol} cells were arbitrarily set to 1. The average and error bars correspond to SEM experiments performed in triplicates are indicated. Statistically significant values using a significance t-Student test are showed, $p<0.015$. (B) Detection of CITED2 protein by western blotting. Protein extracts from Cited2$^{flox/flox}$ mES cells, two days after treatment with Ethanol (Cited^{Ethanol}) or Tamoxifem (Cited2^{4HT}) detection was performed using anti-CITED2 monoclonal antibody and anti-β-tubulin.

4.2.1. Conditional deletion of Cited2 during embryonic stem cell differentiation decreases the number of contractile foci

To evaluate the progression of differentiation from Cited2$^{flox/flox}$ mESC, we observed cell cultures during 12 days after the starting differentiation time (day 0). The time 0 of differentiation refers to the beginning of the differentiation and in fact corresponds to a time point in which ESC are still in a pluripotent state. The observation of the differentiating cells in culture by light microscopy is a simple and efficient way to assess the morphological changes of the cells and to observe the emergence of beating foci. During mESC differentiation, a spontaneously contracting loci indicates that cells have eventually differentiated into mature cardiomyocytes.

Cited2 knockout was triggered at different days during Cited2$^{flox/flox}$ mESC differentiation (treatment with Tamoxifen, 4HT at day 0, 2, 4 and 6) and compared to control cells (treated with Ethanol, vehicle, at the same time points), and we monitored the emergence of beating foci (Figure 4.4A) in Cited2^{4HT} and in Cited2^{Ethanol} control cells.

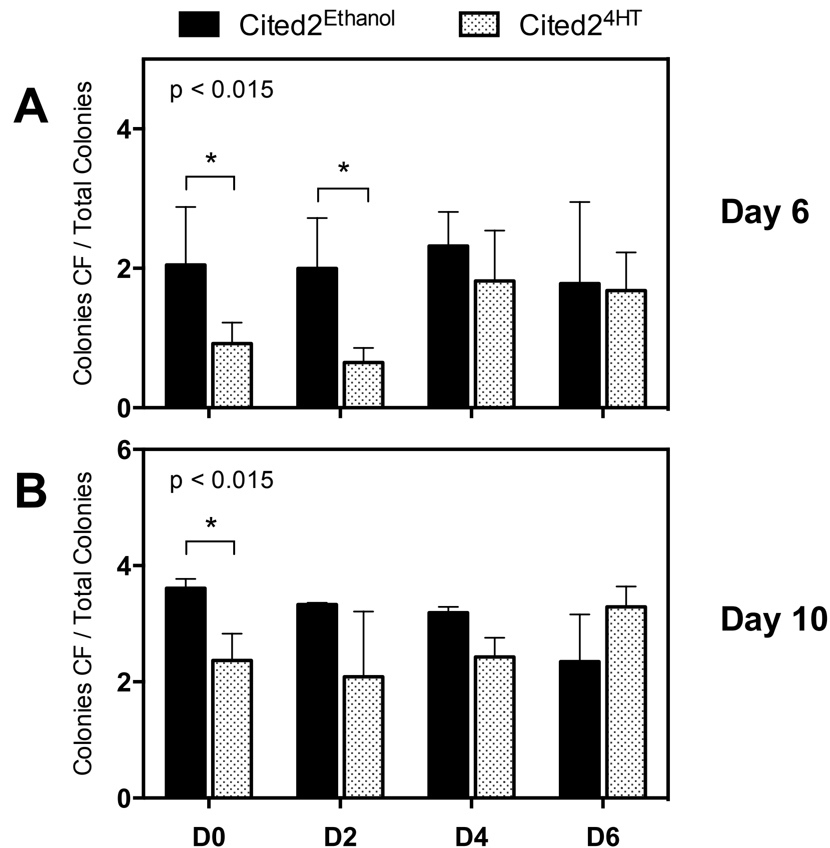


Figure 4.4. Contractile foci number from mES Cited2$^{flox/flox}$ cell cultures treated with vehicle or 4HT at different time points during differentiation.

Number of contractile foci in control cells Cited2^{Ethanol} (black histogram) or Cited2^{4HT} (dotted histogram) at day 6 (A) and day 10 (B). Treatment with ethanol or 4HT was done at the indicated time points of differentiation (D). Colonies that harbored contractile foci (Colonies CF) were counted and normalized to total number of colonies (Total Colonies) present in culture. The error bars correspond to SEM of triplicates. The asterisk (*) corresponds to statistically significant values using a significance t-Student test, $p<0.015$.

The emergence of beating foci in all cell cultures was observed by the 6th differentiation day (Figure 4.4A). We counted the number of contractile colonies that harbor contractile loci, and the total number of contractile loci per plate. We observed a decrease in the number of contractile foci in the Cited2^{4HT} ESC cultures when the knockout was induced by addition of 4HT at day 0 and at day 2 of differentiation in comparison to beating foci counted in the control ESC cultures. No significant differences in the contractile foci number were observed between the control cultures and cultures induced with addition of 4HT at days 4 and 6 of differentiation. By day 10 of differentiation, the number of contractile foci in Cited2^{4HT}, in all conditions, was not significantly different compared to Cited2^{Ethanol} (Figure 4.4B), except for the cells deleted for Cited2 at D0, which still presented a significant decrease in the cardiac cell

lineage differentiation. Together, these results suggest that Cited2-depleted cells were either impaired in their initial commitment to cardiac cell lineage, delayed in their differentiation towards cardiomyocytes or their differentiation was diverted to cells of a different nature.

Moreover, in ESC cultures induced by addition of 4HT at day 0, the Cited2 transcript levels remained significantly lower than in control cells from day 0 to day 5 of differentiation (Figure 4.5). We hypothesized the low expression levels of Cited2 were causing the decrease in contractile foci observed at day 6 of differentiation of $Cited2^{4HT}$ ES cell cultures.

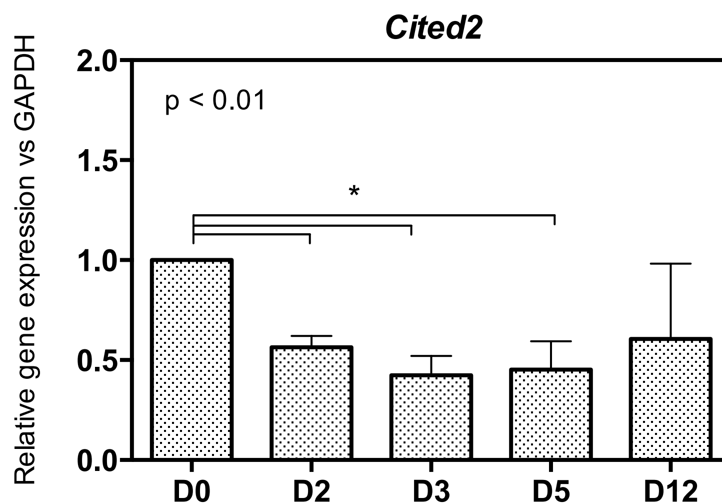


Figure 4.5. Cited2 gene expression in $Cited2^{flox/flox}$ mESC cultures treated with vehicle or 4HT at day 0. Relative gene expression of *Cited2* in $Cited2^{4HT}$ cultures normalized against gene expression in $Cited2^{Ethanol}$ cultures, and compared to day 0 (starting differentiation) that was arbitrarily set to 1. Differentiation days (D0 to D12) are indicated. The average and error bars correspond to SEM experiments performed in triplicates are indicated. Statistically significant values using a significance t-Student test are showed, $p<0.01$.

4.2.2. CITED2 knockout at the onset of embryonic stem cell differentiation impairs mature cardiac gene expression

In order to confirm that the reduction of the contractile foci number in cultures of ES cells Cited2-depleted was related with the down-regulation of characteristic mature cardiomyocyte genes, we sought for the expression of alpha-myosin heavy chain, *αMHC*, and cardiac troponin T, *Tnt*. If the gene expression of these two markers would be down-regulated, it might be due to a shift in cell commitment, probably by enhancing the gene expression of others mature cell markers derived from endoderm

and/or ectoderm lineage. For that reason we also tested the expression of hepatocyte (which are derived from the endoderm) specific gene markers, such as *FoxA2* and *Afp* [14], and the expression of markers from neural cells (derived from the ectoderm), such as *Nestin* and *β-III-Tubulin*.

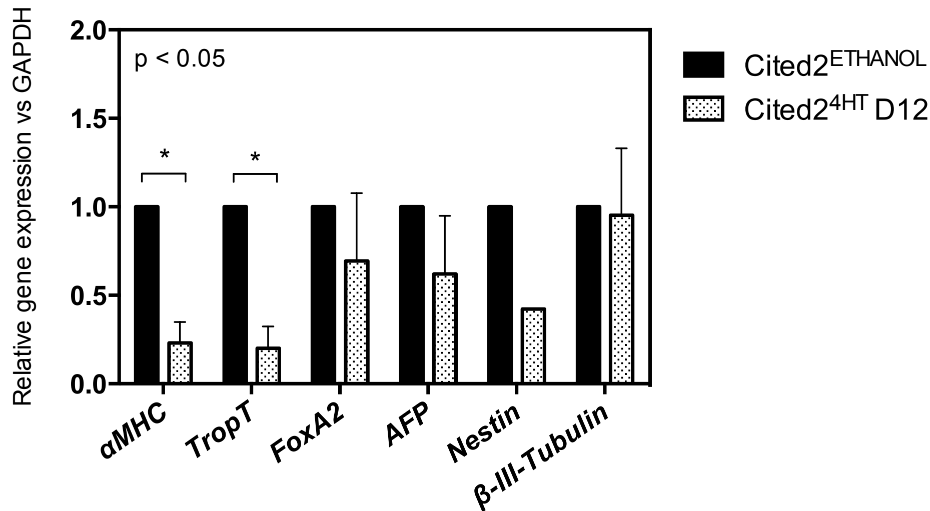


Figure 4.6. Mature gene markers expression at day 12 of differentiation in Cited2$^{flox/flox}$ cultures treated with vehicle or 4HT at day 0.
Cited2^{Ethanol} (black histogram) or Cited2^{4HT} cells (dotted histogram) analyzed at day 12 of differentiation. The relative fold change expression was compared to the values from each Cited2^{4HT} cultures normalized against gene expression in Cited2^{Ethanol} cultures. The error bars correspond to SEM of triplicates. The asterisk (*) correspond to statistically significant values using a significance t-Student test, $p<0.05$.

We performed a transcript expression analysis from cells treated with 4HT and Ethanol at the beginning of differentiation (day 0) and collected at day 12 of differentiation (Figure 4.6). We observed lower levels of mature cardiomyocyte specific transcripts in Cited2^{4HT} ESCs, when compared to control cells. This observation is in agreement with the reduction in the number of contractile foci observed in Cited2^{4HT} ESCs at day 6 and day 10 of differentiation (Figure 4.4). Moreover, a decrease in gene expression of liver and neural gene markers was also observed in Cited2^{4HT} ESCs in comparison to control ESCs, but these differences did not reach statistical significance. The shift in cell commitment appeared not be the case for the delay in contractile foci number. Overall, these results confirmed that Cited2-depleted ESCs were less prone to fully differentiate into mature cardiomyocytes. Since, the decrease in cardiac fate differentiation of Cited2-depleted ESCs only occurred when Cited2 expression was knocked out at Day 0 or Day 2 of differentiation, we hypothesized that Cited2 is either involved in early events of ESC differentiation towards differentiation or required for the maturation of premature cardiomoyocytes into beating cardiac cells. Alternatively,

Cited2-depleted cells might undergo an increased cell death, as we have observed for Cited2-depleted ESC maintained in pluripotent conditions (*Manuscript submitted*).

On their path towards differentiation into mature cardiac myocytes, ESCs go through an intermediate stage of cardiac progenitors cells[30, 130]. During this stage, the cardiac progenitors express *Nkx2.5*, *Tbx5*, *Isl1* and *Gata4* transcription factors, which together induce the expression of early cardiac muscle specific genes, such as *Mef2c*. To verify whether, more cardiac progenitor markers were expressed at day 12 of differentiation in ESCs depleted for Cited2 than the control cells, we performed a qRT-PCR (Figure 4.7). The relative transcript levels of *Nkx2.5*, *Isl1, Mef2c* and *Gata4* at day 12 of differentiation were similar in Cited2^{4HT} and control ESCs, while *Tbx5* gene marker presented a significantly lower expression level in Cited2-depleted ESCs. These observations suggested that Cited2-depletion might not markedly affect the transcription of gene markers of cardiac progenitor cells at day 12.

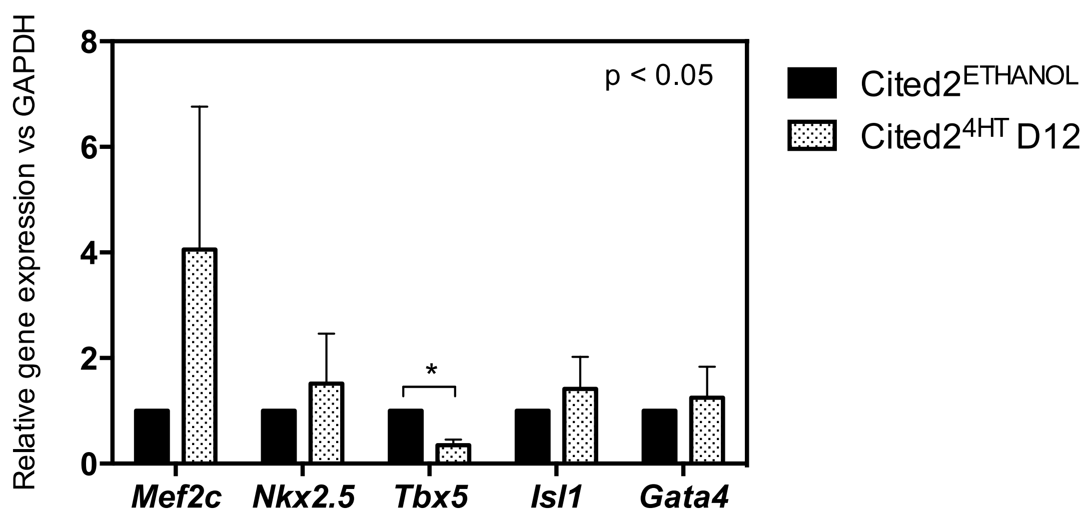


Figure 4.7. Cardiac progenitor gene markers at day 12 in differentiated Cited2$^{flox/flox}$ cultures treated with vehicle or 4HT at day 0.
Cited2^{Ethanol} (black histogram) or Cited2^{4HT} cells (dotted histogram) were analyzed at day 12 of differentiation. The error bars correspond to SEM of triplicates. The (*) asterisk correspond to statistically significant values using a significance t-Student test, $p<0.05$.

T-box transcription factor 5 (Tbx5) marks the transition from primordial muscle type to chamber myocardium[131]. During this transition, Tbx5 acts as an activator genes required for intercellular coupling such as connexin 40 (*Cx40*) as well as other genes such as atrial natriuretic factor (*Nppa*)[132]. In addition, *Tbx5* has been shown to be required for cardiac function and for full differentiation into contracting

cardiomyocytes from reprogramming cardiac fibroblast[44]. To verify, whether Tbx5-target genes were affected in Cited2-depleted cultures at day 12 of differentiation, we performed a transcript level assessment by qRT-PCR (Figure 4.8). Our results indicated a concomitant reduction in the expression levels of *Tbx5* and *Nppa* transcripts in Cited2^{4HT} cultures, while no difference was observed in *Cx40* expression (Figure 4.8).

Next, we sought for cell cycle gene expression markers, *cyclin-D1* and *cyclin-D2*, since Tbx5 regulates cardiac cell cycle associated G_1/S-phase proteins in early heart development[133]. No significant differences in *CycD1* (*Cyclin-D1*) transcripts levels were observed in Cited2^{4HT} ES cells cultures in comparison to control cells, while *CycD2* (*Cyclin-D2*) expression appeared to be increased by approximately 3 folds in Cited2^{4HT} cultures.

Taken together, these data suggest that the delay in the appearance of contractile foci in Cited2^{4HT} cultures might be caused, at least in part, by the reduction in *Tbx5* gene expression.

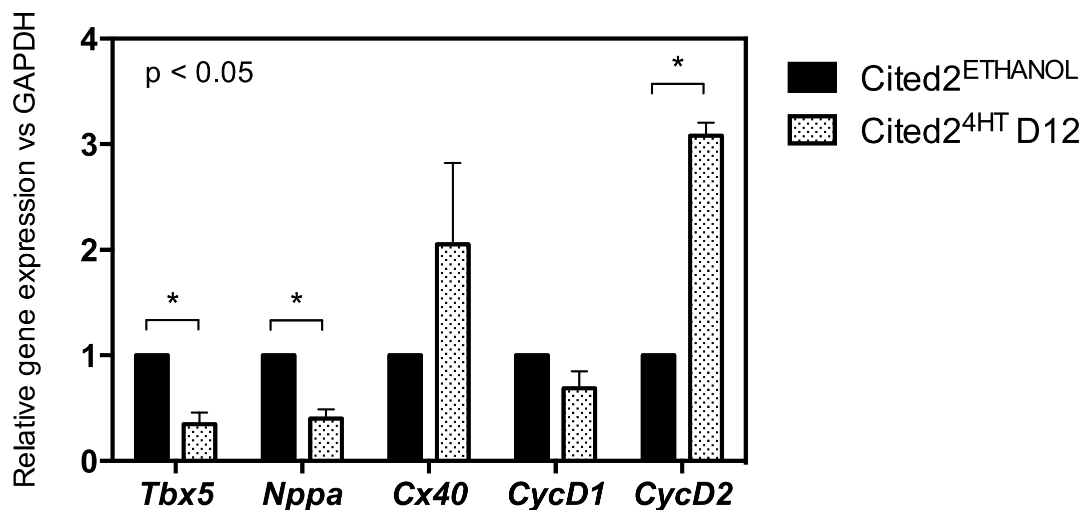


Figure 4.8. Analysis of Tbx5 and downstream Tbx5 target genes expression in Cited2$^{flox/flox}$ ESC cultures treated with vehicle or 4HT at day 0.
Cited2^{Ethanol} (black histogram) or Cited2^{4HT} cells (dotted histogram) were analyzed at day 12 of differentiation. The error bars correspond to SEM of triplicates. The (*) asterisk correspond to statistically significant values using a significance t-Student test, $p<0.05$.

4.2.3. Cited2 is important for Mesoderm initiation stage in embryonic stem cells

4.2.3.1. Initiation of mesoderm commitment in embryonic stem cells

In order to determine whether *Cited2* was involved in the early stages of cardiac differentiation, we sought to determine in ESC differentiation whether the mesoderm induction or early cardiac specification stages were affected by Cited2 knockout. We performed the Cited2 depletion in Cited2$^{flox/flox}$ ESCs by treatment with 4HT at day 0, and collected the cell samples at early stages of differentiation (day 2, day 3 and day 5 after 4HT treatment).

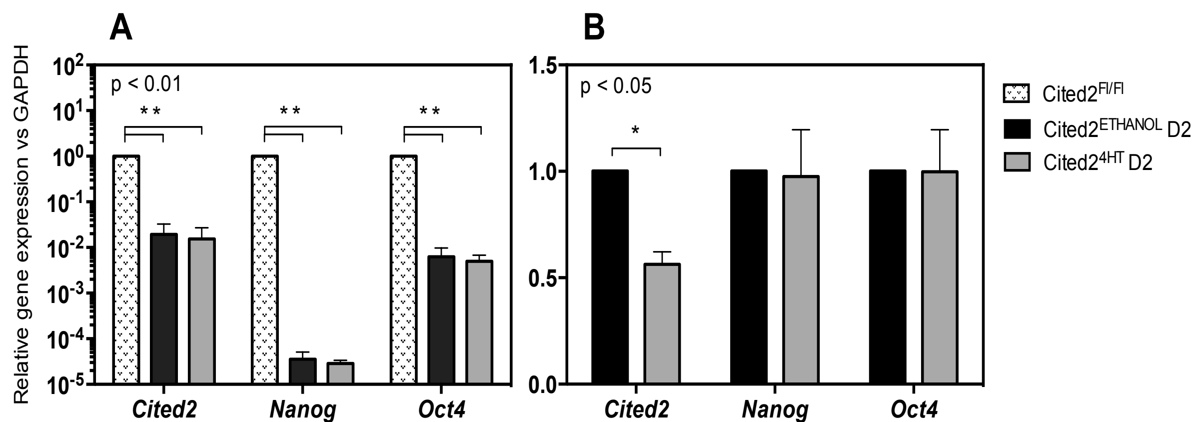


Figure 4.9. Pluripotency gene expression in pluripotent Cited2$^{flox/flox}$ mESC (day 0) and in differentiated cultures (day 2).
(A) *Cited2*, *Nanog* and *Oct4* gene expression in pluripotent Cited2$^{flox/flox}$ mESC at day 0 (dotted histogram) an in Cited2^{4HT} cells at differentiation day 2 (gray histogram). (B) *Cited2*, *Nanog* and *Oct4* gene expression in Cited2^{Ethanol} cells (dark histogram) comparing to Cited2^{4HT} cells (gray histogram), at day 2 of differentiation. The error bars correspond to SEM of triplicates. Statistically significant values using a significance t-Student test are showed, *p<0.01 and **p<0.05.

Pluripotency-related genes are markedly downregulated within the first 24-48 hours of differentiation. Thus, to verify whether this initial step of differentiation, which consists in switching off the expression of pluripotency-related genes, was occurring normally, we assessed the expression of *Nanog* and *Oct4*. As expected, we observed a reduction of their expression, as early as day 2, that was not perturbed by Cited2 depletion (Figure 4.9). Interestingly, Cited2 expression was also markedly reduced at days 2 and 3 in the control cells, suggesting that like Nanog and Oct4, Cited2 expression is also down-regulated within the first hours of differentiation of ESC (Figure 4.9A). Even though, Cited2 expression decreased within the first 48 hours with the expected normal pattern in the differentiation course of ESCs, the treatment of Cited2$^{flox/flox}$ ESCs

with 4HT led to further a reduction of Cited2 expression by approximately 2 fold, indicating the depletion of Cited2 by knockout (Figure 4.9 and 4.10).

The earliest known event influencing differentiation of the ICM into its two derivatives: the epiblast and the primitive endoderm, is linked to the expression of the transcription factor Gata4, which promotes the emergence of the primitive endoderm. Gata4 and Sox17 have been associated in the normal process for down-regulation of *Nanog* expression, where the accumulation of Sox17 in mESC leads to engage the differentiation program[134]. *Sox17* was shown to be essential for the specification of cardiac mesoderm during mESC differentiation[135].

We assessed the expression of *Nanog*, *Gata4* and *Sox17* genes in $Cited2^{Ethanol}$ and $Cited2^{4HT}$ cultures and observed no differences resulting from Cited2-depletion (Figure 4.10), suggesting that the early steps of differentiation do not require Cited2.

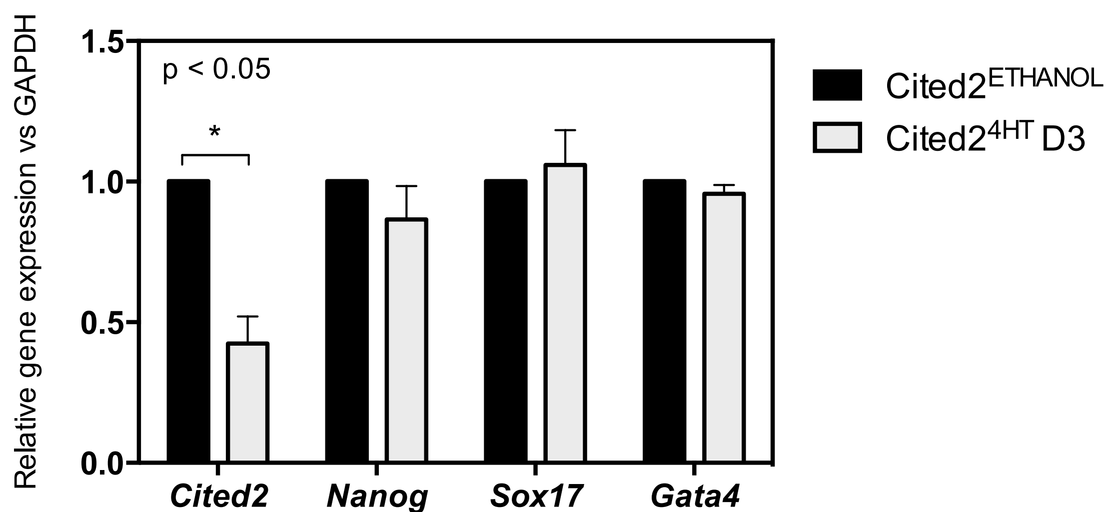


Figure 4.10. Mesendoderm markers at day 3 of differentiation in $Cited2^{flox/flox}$ cultures treated with vehicle or 4HT, at day 0.
The relative fold change expression was presented, comparing the values from each culture, $Cited2^{4HT}$ (light histogram), to its control, $Cited2^{Ethanol}$ (black histogram). The error bars correspond to SEM of triplicates. The asterisk (*) correspond to statistically significant values using a significance t-Student test, $p<0.05$.

4.2.3.2. Mesoderm induction in embryonic stem cells

During early gastrulation, distinct subpopulations of mesoderm cells are induced at different primitive streak regions, whereas the definitive endoderm forms from the anterior region of primitive streak. The mesoderm induction is the first step towards cardiac cell fate, and is characterized by the expression of *T*/Brachyury in the primitive

streak mesoderm and mesendoderm, that induces the expression of *Mesp1* [136, 137]. Mesp1 expression emerges subsequently to Brachyury as the earliest molecular marker of cardiac precursor cells which is essential for cardiac myogenesis in committed mesoderm cells[138]. *Mesp1* drives cardiac lineage commitment[123, 139] and is expressed transiently in cells of early mesoderm[140] from which the endoderm and mesoderm segregate afterwards.

We sought for the relative expression of early mesoderm markers, such as *Brachyury* and *Mesp1*, in Cited2^{4HT} differentiated and control cell cultures, Cited2^{Ethanol} (Figure 4.11).

The relative expression level for Brachyury at day 3 was markedly decreased in Cited2^{4HT} (Figure 4.11). Moreover, *Mesp1* relative expression dropped by day 3 in Cited2^{4HT}, compared to Cited2^{Ethanol}.

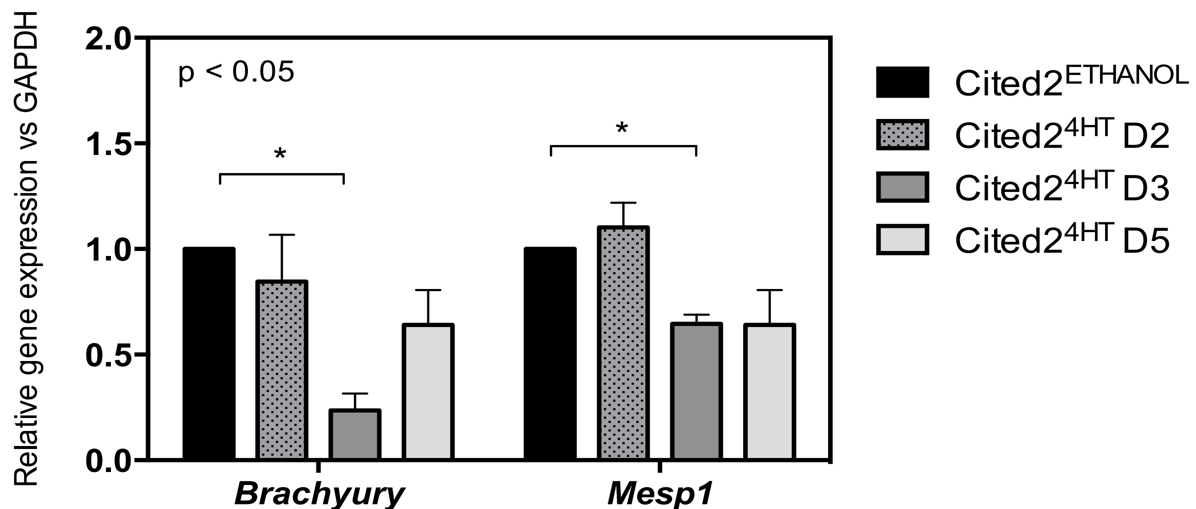


Figure 4.11. Mesoderm (Brachyury and Mesp1) gene expression levels in Cited2$^{flox/flox}$ mESC cultures treated with vehicle or 4HT, at day 0.
Relative gene expression levels at day 2 (dotted histogram), day 3 (gray histogram) and day 5 (light histogram) of differentiation. The relative gene expression in Cited2^{4HT} (dotted histogram) was normalized against gene expression in Cited2^{Ethanol} (black histogram), and compared vs day 0 that was arbitrarily set to 1. The error bars correspond to SEM of triplicates. The asterisk (*) correspond to statistically significant values using a significance t-Student test, $p<0.05$.

Next, we assessed the expression of gene markers for mesendodermal induction (Figure 4.12). The mesendoderm gives rise to both mesoderm and endoderm, from which the definitive endoderm arises, and is characterized by the co-expression of goosecoid gene, *Gsc,* and *Brachyury*[141]. The Goosecoid (*Gsc*) gene is a direct target of Brachyury, and its expression is positively regulated by *Nodal* and *Activin A*[141]. It has been reported that the expression levels of *Nodal*, a member of the transforming growth

factor-β-related TGF-β family, increase during the early stages of differentiation and then are shut down, when the activation of the *Activin A* expression starts[142]. *Activin A* is also member of the TGF-β family, activating the same signaling cascade activated by Nodal. *Activin A* has been reported to be important for efficient cardiac differentiation.

We observed that *Gsc* expression was significantly decreased at day 3 in Cited2^{4HT}, and no differences were found in *Nodal* and *Activin A* expression. This data suggests that the depletion of Cited2 in ES cells when they are still in a pluripotent state affects their efficient transition to mesendodermal cells. Since *Nodal* and *Activin A*, which activate Gsc expression, are not affected by Cited2-depletion cells, Cited2 might contribute to the direct or indirect regulation of *Gsc* expression.

Taken together, these results suggest that Cited2 expression is needed at the mesoderm induction for cardiac specification transition.

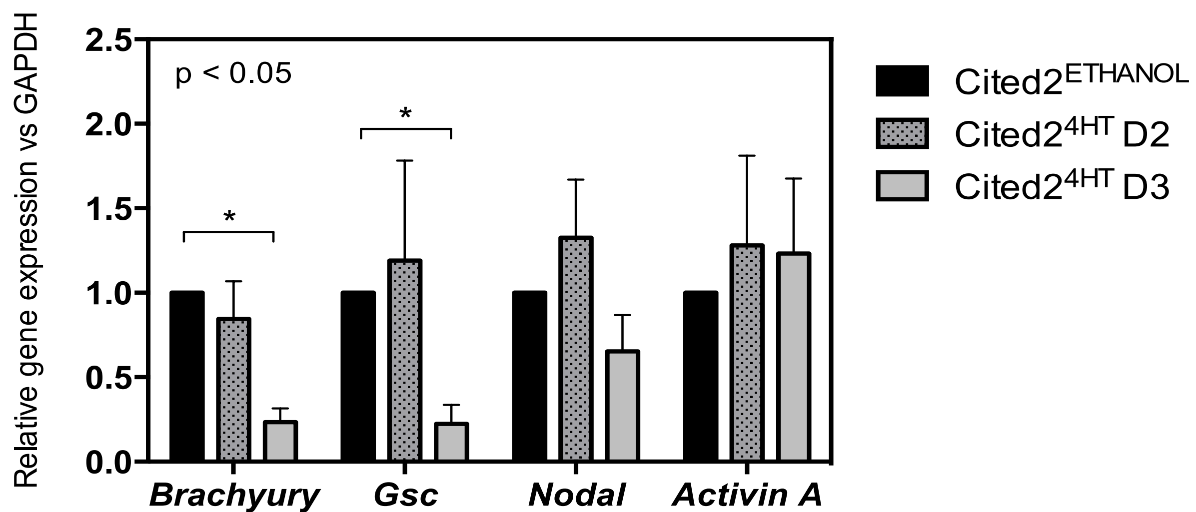


Figure 4.12. Mesendoderm gene expression in Cited2$^{flox/flox}$ cultures treated with vehicle or 4HT, at day 0. *Brachyury*, *Gsc*, *Nodal* and *Activin* relative gene expression are presented at day 2 (dotted histogram) and 3 (grey histogram) of differentiation. The relative gene expression in Cited2^{4HT} (dotted histogram) was normalized against gene expression in Cited2^{Ethanol} (black histogram), and compared vs day 0 that was arbitrarily set to 1. The error bars correspond to SEM of triplicates. The asterisk (*) correspond to statistically significant values using a significance t-Student test, p<0.05

4.3. CITED2 gain-of-function experiments in embryonic stem cells

The observations obtained from Cited2-knockout experiments in early mESC differentiation suggested that Cited2 is mostly involved in the initial steps of cardiac cell lineage differentiation. Thus, to gain further insights on the role of Cited2 in this early process, we performed gain-of-function experiments in mESC line E14/T stably

transfected with the pPyCAGIP-flagCited2 vector (FC2), described elsewhere[114]. FC2 overexpresses the human CITED2 peptide flag tagged at its N-terminal domain (Figure 4.13). pPyCAGIP-derived plasmids are able to replicate in an episomal form in E14/T cells and confer a puromycin resistance to the cells.

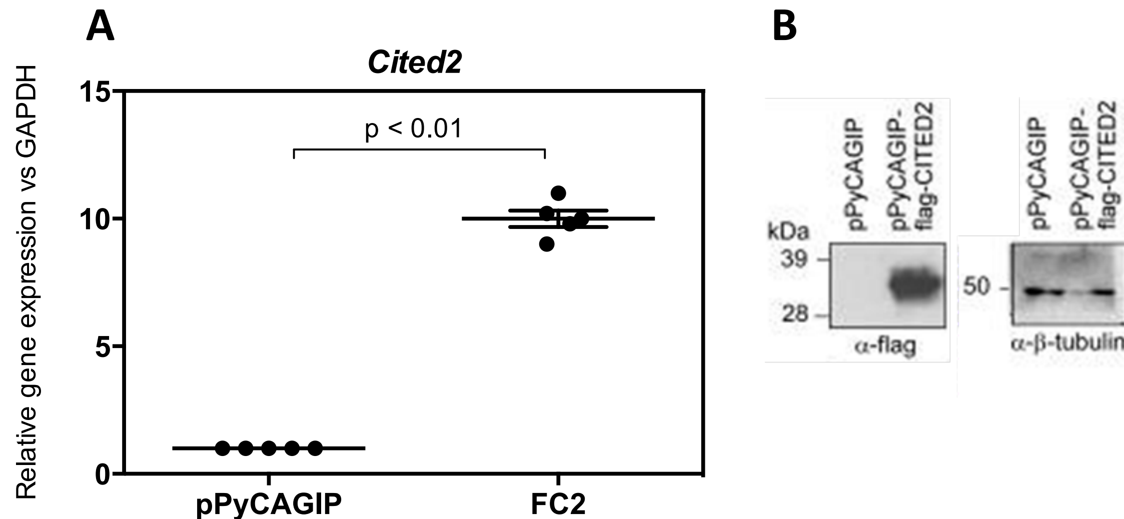


Figure 4.13. Gene expression and protein levels in pluripotent E14/T ES overexpressing FlagCITED2.
(A) Total RNA extraction from pluripotent E14/T ES cells were transfected with pPyCAGIP-flagCITED2 (FC2) or the control vector (pPyCAGIP). Gapdh was used as a control. The levels of Cited2 expression in cells transfected with pPyCAGIP were arbitrarily set to 1. The average and error bars correspond to SEM of n=5 experiments performed and significant values using a significance t-Student test are presented, p<0.01. (B) *Detection of CITED2 protein by western blotting.* Protein extracts from E14/T cells transfected with pPyCAGIP-flagCited2 or the control vector pPyCAGIP, using anti-CITED2 monoclonal antibody, loading control, anti-b-tubulin monoclonal antibody.

4.3.1. Cited2 gain-of-function during embryonic stem cell differentiation enhances the number of contractile foci

Differentiation cultures were performed with mES cell line E14/T stably transfected with the pPyCAGIP-flagCited2 (FC2) and its control vector (pPyCAGIP) and maintained with (+) or without (-) puromycin (resistance cassette). Interestingly, ES cultures forced to maintain FlagCITED2 overexpression by the presence of puromycin in the medium, formed EB when they were seeded for differentiation but they fail to undergo the normal differentiation process when compared to cells differentiated in the absence of puromycin (Figure 4.14).

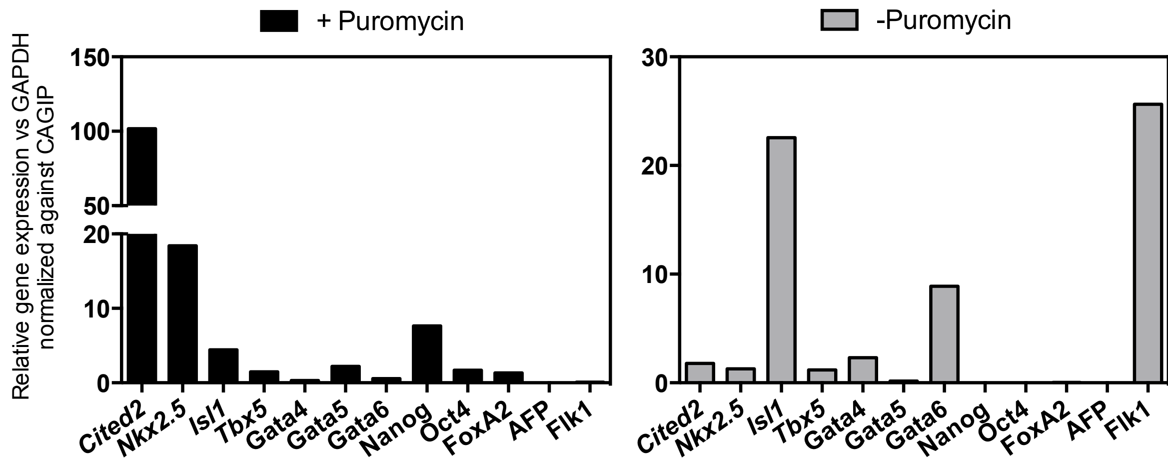


Figure 4.14. Gene expression in FlagCITED2 overexpression ESC differentiated cultures maintained with puromycin.

Relative gene expression for *Cited2*, cardiac progenitor (*Nkx2.5*, *Isl1*, *Gata4-6*), pluripotency (*Nanog*, *Oct4*) and endodermal gene markers (*FoxA2*, *Afp*, *Flk1*), in cultures with puromycin (black histogram) or without puromycin (grey histogram). Gene expression was normalized against Gapdh expression and compared with E14T-pPyCAGIP gene expression; *n*=1.

After 12 days of differentiation, the FC2 cultures without the addition of puromycin were capable of differentiate into cardiomyocytes, unlike the FC2 cultures that remained mostly with EB morphology and did not adhere to the plate as if their differentiation was delayed or prevented. In order to assess the differentiation progression of the differentiating cells, we sought for pluripotency and mature gene markers of FC2 and control cells differentiated in medium with or without puromycin for 12 days (Figure 4.14). We observed that FC2 cells maintain with puromycin in the medium expressed very high amounts of flag-CITED2 transcripts in comparison to control cells transfected with pPyCAGIP and cells maintained in culture without puromycin, as expected. In addition, the expression of the pluripotency markers, as Nanog and Oct4 was also detected in the cells expressing high levels of Cited2, suggesting that the forced expression of Cited2 at high levels, either prevents ESCs from an efficient differentiation or preserves a pool of pluripotent cells amongst the population of cell that are differentiating. Interestingly, some cardiac progenitor gene markers, such as *Nkx2.5*, *Tbx5* and *Gata5* were also upregulated in cultures that maintains high levels of Cited2 expression in the presence of puromycin. Interestingly, the initial overexpression of flag-CITED2 in pluripotent E14/T cells also promoted high expression levels of *Flk1*, *Isl1* and *Gata6* at day 12 of differentiation in the absence of puromycin.

Together, these observations suggested that FC2 cells expressing extremely high levels of flag-CITED2 transcript are impaired in the differentiation abilities and retain expression of genes involved in the maintenance of pluripotency (*Nanog* and *Oct4*), or early differentiation into cardiac progenitors (*Nkx2.5*, *Tbx5* and *Gata5*), and even early mesendodermal/endodermal markers such as Foxa2.

Since the presence of puromycin in the culture medium during the differentiation process, leading to high levels of flag-CITED2 expression, blocked or delayed the differentiation of E14/T cells in standard hanging drop conditions, we have decided to investigate the effects of Cited2-gain of function in E14/T cells when differentiated in the absence of puromycin. We analyzed the cultures at days 0, 3, 6 and 12, post-differentiation, time 0 of differentiation referring to the induction of differentiation and corresponings in essence to pluripotent cells.

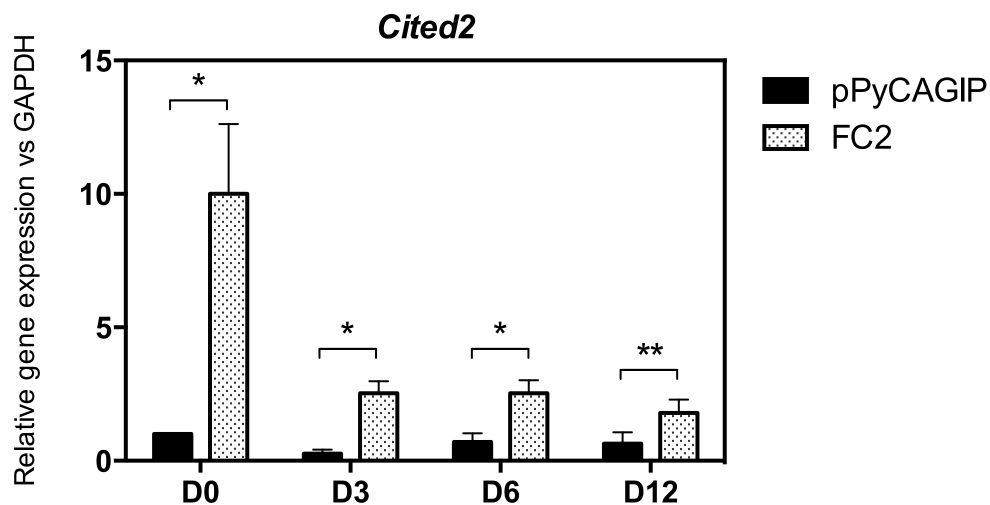


Figure 4.15. Cited2 expression during mESC differentiation of E14/T in gain-of-function Cited2 experiments. Cited2 transcripts during ESC differentiation in control cells (pPyCAGIP, black histogram) or pPyCAGIP-flagCITED2 (FC2, dotted histogram). Differentiation days (D) are indicated. Gene expression was normalized against Gapdh expression and compared with pPyCAGIP gene expression at D0. The error bars correspond to SEM of triplicates and significant values using a significance t-Student test were calculated, *p<0.01 and **p<0.05.

Through all the differentiation process, we observed an increase of Cited2 mRNA levels in the cells overexpressing Cited2 compared to the control cells (Figure 4.15).

In order to determine the effect of Cited2 overexpression in E14/T cardiac differentiation, we monitored and compared the emergence of contractile foci in mESCs overexpressing Cited2 (FC2) and control cells (pPyCAGIP).

We observed the emergence of contractile foci in cell cultures with flag-CITED2 overexpression, at day 7, whereas the first beating foci in control cells were only observed 3 days later (Figure 4.16). Beating foci indicated the presence of cardiomyocyte cell commitment during the differentiation program, so these results suggest that CITED2 overexpression promotes cardiomyogenesis.

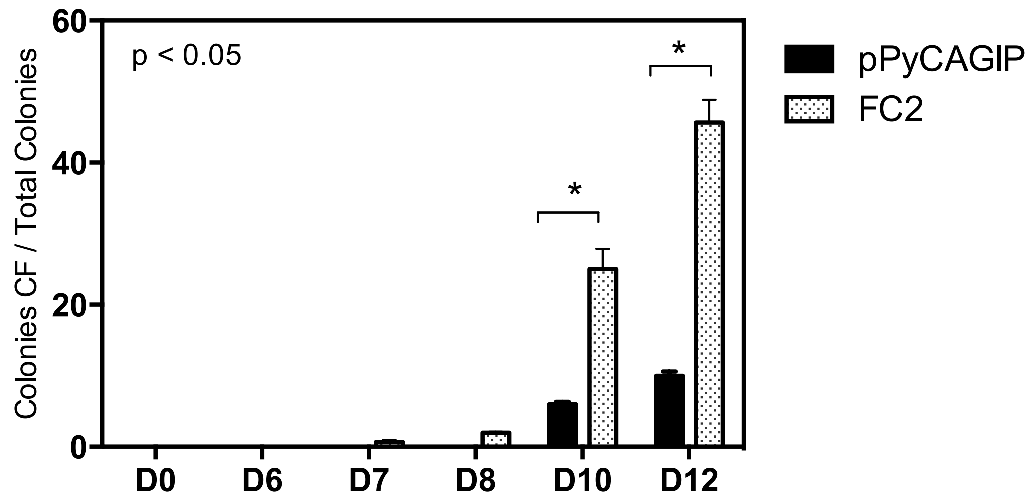


Figure 4.16. Contractile foci in ESC differentiated in gain-of-function Cited2 experiments.
Control cells pPyCAGIP (black histogram) or FC2 (dotted histogram) at the indicated days of differentiation (D). Colonies that harbor contractile foci (Colonies CF) were counted and normalized to total number of colonies (Total Colonies) in culture. The error bars correspond to SEM of triplicates and significant values using a significance t-Student test were calculated, *p<0.05.

4.3.2. Mature cardiac genes are enhanced in Cited2 gain-of-function embryonic stem cell cultures

Since we have found that an overexpression of CITED2 accelerates the appearance of beating foci in E14/T differentiation cultures, we asked whether Cited2 could also induce the expression of characteristic genes of mature cardiac cells.

Gene expression analysis of FC2 cultures overexpressing flag-CITED2 showed an elevated expression of genes normally expressed in mature cardiomyocytes (Figure 4.17). Specifically, we have detected an elevated gene expression of *α-myosin heavy chain* (*α-MHC*) and *Troponin-T* (*Tnt*) in cells with flag-CITED2 overexpression from day 6. These observations are in agreement with the early appearance of beating foci in cultures overexpressing CITED2.

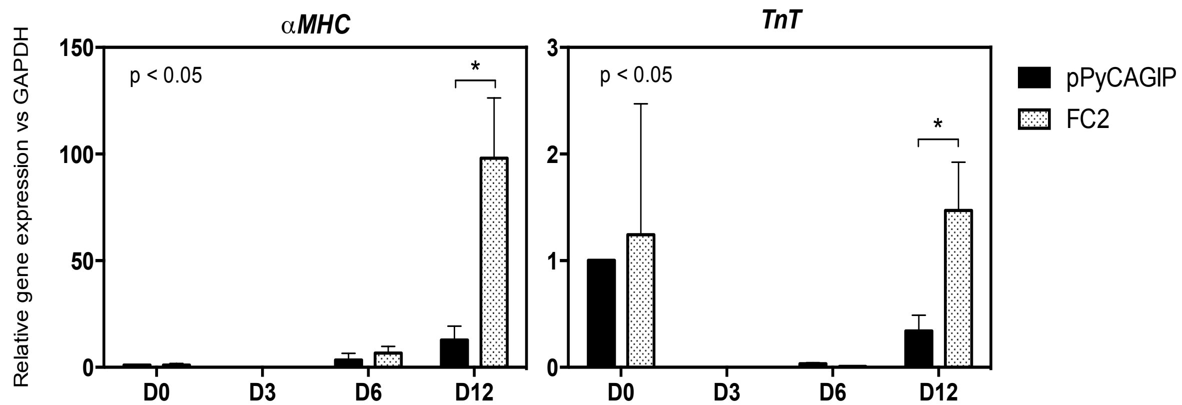


Figure 4.17. Mature cardiac gene markers expression at day 12 of differentiation in gain-of-function Cited2 experiments.
Relative gene expression of α-myosin heavy chain (*α-MHC*) and Troponin-T (*Tnt*) in control cells pPyCAGIP (black histogram) and FC2 cells (dotted histogram) were analyzed at different differentiation days (D). Gene expression was normalized against Gapdh expression and compared with pPyCAGIP gene expression at D0. The error bars correspond to SEM of triplicates. The (*) correspond to statistically significant values using a significance t-Student test, $p<0.05$.

4.3.3. Mesendoderm commitment induced by Cited2

4.3.3.1. Mesoderm induction by Cited2

In Cited2-depletion experiments, we have observed that ESCs differentiation might be affected in the early steps of ESCs commitment to mesoderm cells, since the expression of markers such as *Brachyury*, *Gsc* and *Mesp1* was reduced in Cited2-konckout cells. Thus, to verify whether flag-CITED2 overexpression could also influence early stages of ESCs differentiation during cardiac mesodermal program, we analyzed the expression of mesendodermal marker genes (Figure 4.18).

Mesoderm induction begins with the activation of Nodal signaling pathway, which precedes the expression of the mesendodermal marker *Brachyury*. Moreover, the expression of mesendodermal markers such as *Brachyury* and *Gsc* has been shown to be transient and to peak at day 3-4 after the beginning of the differentiation and then abruptly decreases at day 5-6[141].

Unexpectedly, both pPyCAGIP and FC2 cultures presented similar gene expression levels for early mesodermal markers *Brachyury* and *Gsc* (Figure 4.18).

On the other hand, *Nodal* expression was significantly decreased in FC2 cultures in pluripotent stem cells (day 0), although by day 3 the differences disappeared (Figure 4.18). Surprisingly, by day 6 of differentiation, the *Nodal* expression was higher in FC2 than in control conditions (Figure 4.18).

It has been reported that around day 6, the expression of Nodal is switched off, but the expression of Activin A is activated at the same time and will activate the same signaling cascade to promote cardiac differentiation[142]. Thus, according to our results the sustained expression of *Nodal* at day 6 in cells overexpressing CITED2 could act to activate or maintain switched on the TGF-β/Nodal signaling cascade and promote cardiac differentiation.

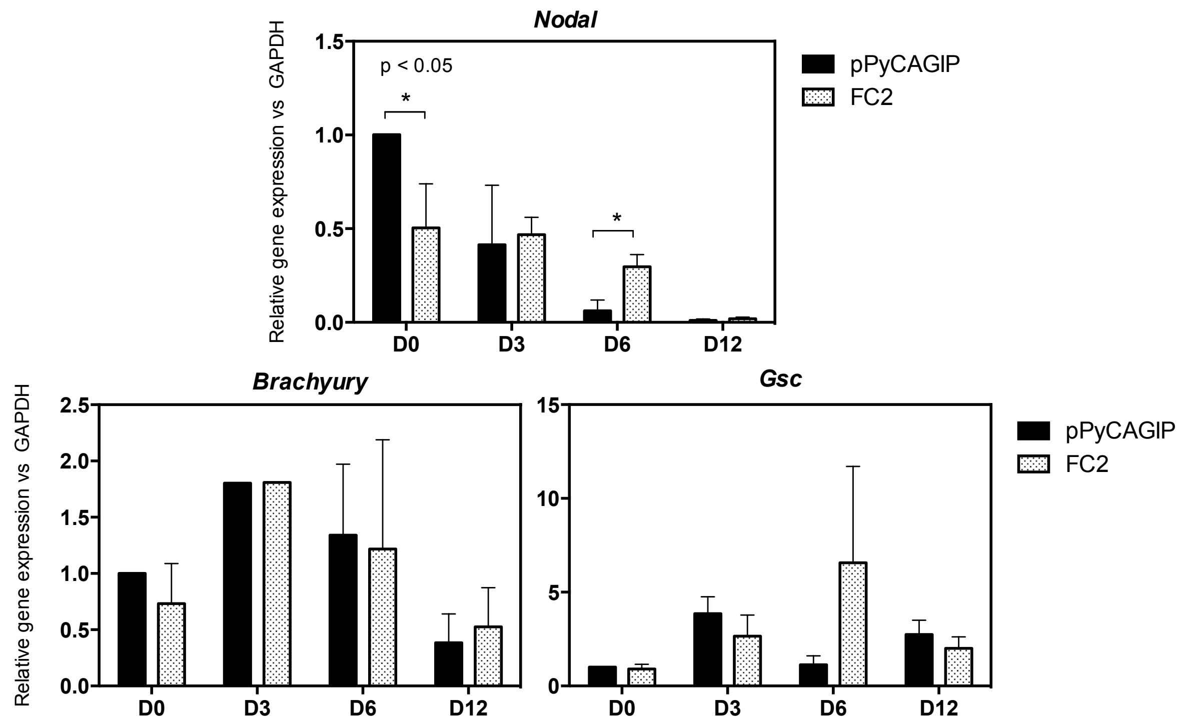


Figure 4.18. Gene expression of mesodermal markers (Gsc, Brachyury) and FGF-β ligand (Nodal) throughout differentiation, in gain-of-function Cited2 experiments.
Control cells pPyCAGIP (black histogram) or FC2 (dotted histogram), and differentiation days (D) are indicated. Gene expression was normalized against Gapdh expression and compared with pPyCAGIP gene expression at D0. The error bars correspond to SEM of triplicates and significant values using a significance t-Student test were calculated, *p<0.05.

Furthermore, in FC2 cultures we observe significant differences in *Pixt2c* and *Hand1* gene expressions at the end of differentiation (Figure 4.19). During embryonic development, *Nodal* is directly required for rightward looping of the heart, whereas *Pitx2c*, a gene which is downstream of *Nodal*, regulates the atrial left–right identity and the rotation of the arterial pole of the heart which is necessary for the correct alignment of the aorta and pulmonary trunk with the left and right ventricles[62]. *Hand1* expression is restricted to the outer curvature of the left ventricular (LV) and OFT when the heart starts to looping[143]. Overall, our observations suggest that the cardiomyocytes

originated in FC2 cultures might present a left ventricular phenotype, but further analysis are required to confirm this hypothesis

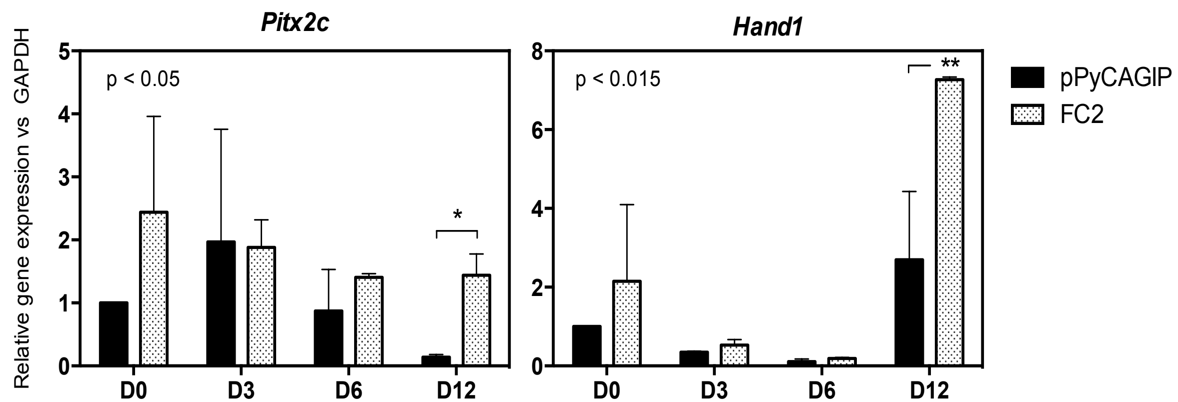


Figure 4.19. Relative gene expression Pitx2c and Hand1 in gain-of-function Cited2 experiments.
Control cells pPyCAGIP (black histogram) or FC2 (dotted histogram), and differentiation days (D) are indicated. Gene expression was normalized against Gapdh expression and compared with pPyCAGIP gene expression at D0. The error bars correspond to SEM of triplicates and significant values using a significance t-Student test were calculated, *p<0.05 and **p<0.015.

4.3.3.2. Endoderm induction by Cited2

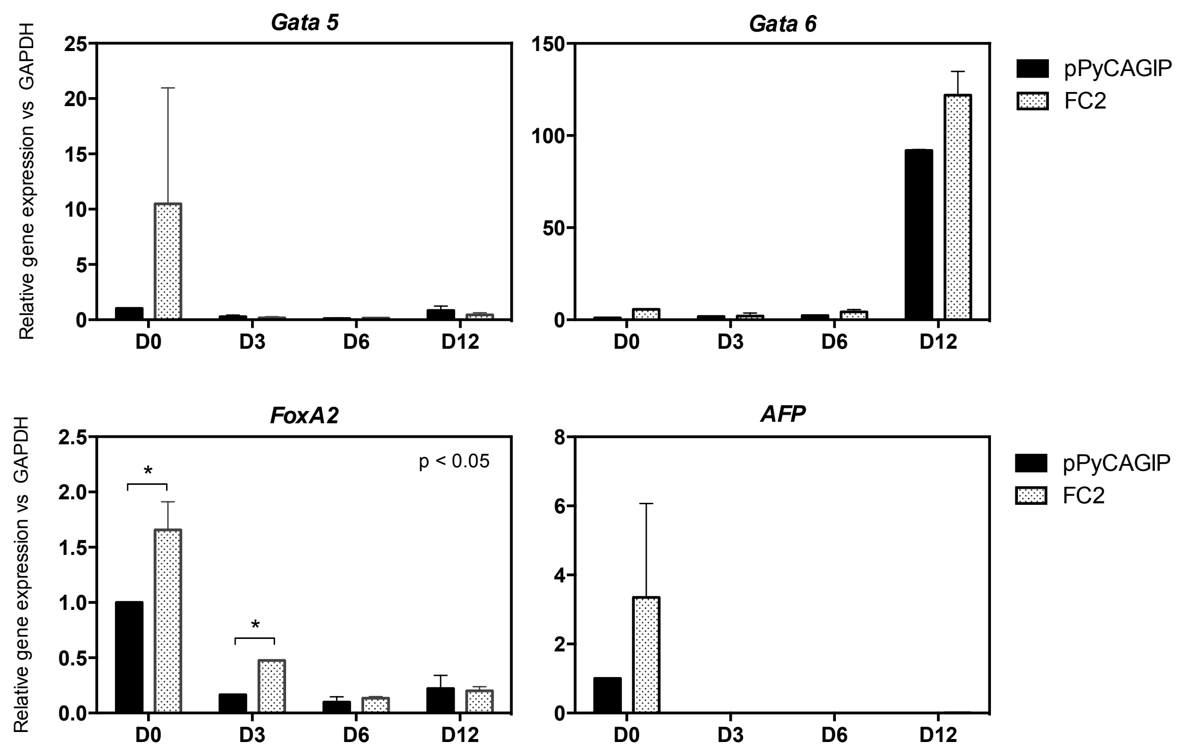


Figure 4.20. Endoderm related markers during ESC differentiation in in gain-of-function Cited2 experiments.
Relative gene expression levels of *Gata5* and *Gata6* (upper panels), *FoxA2* and *AFP* (lower panels) are shown. Control cultures (dark histogram) and FC2 cultures overexpressing Flag-Cited2 (dotted histogram) throughout differentiation. Gene expression was normalized against Gapdh expression and compared with pPyCAGIP gene expression at D0. The error bars correspond to SEM of triplicates and statistically significant values using a significance t-Student test were calculated, *p<0.05.

The visceral endondermal cells signals to epiblast-derived cells, leading to the proper position of the heart. GATA factors 4, 5 and 6 share a common role in the specification of both endoderm and mesoderm[144]. Although, depletion of Gata5 was dispensable for development in mouse, Gata4 and Gata6 depletion results in mice lacking the heart[145]. Furthermore, the Forkhead box a2 (*FoxA2*) is required for endoderm development and normal left-right asymmetry in mouse[146], and the α-1-fetoprotein (*AFP*) is expressed in the visceral endoderm[147].

Among the four-endoderm gene markers analyzed, only *FoxA2* presented significant differences in FC2 cultures at the onset, as well as by day 3 of differentiation (Figure 4.20). Moreover, the higher expression levels for *FoxA2* were not related with high expression of *AFP* as previously reported[148], suggesting that CITED2 overexpression might promote the early endoderm differentiation (progenitors) and not the later stages of endodermal-derived cells. Taken together, the endoderm gene expression profiles suggested that the overexpression of Cited2 might also promote cardiac differentiation through non-cardiac mechanism.

4.3.3.3. Vascular induction by Cited2

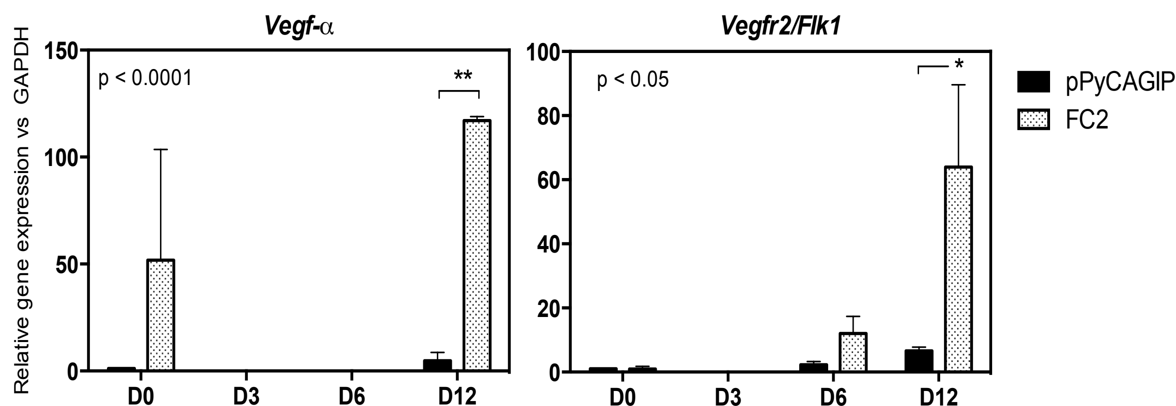


Figure 4.21. Vascular cell markers, Vegf-α and Flk1, during ESC differentiation in gain-of-function Cited2 experiments.
Control cells pPyCAGIP (black histogram) or FC2 (dotted histogram), and differentiation days (D) are indicated. Gene expression was normalized against Gapdh expression and compared with pPyCAGIP gene expression at D0. The error bars correspond to SEM of triplicates and statistically significant values using a significance t-Student test were calculated, $**p<0.001$ and $*p<0.05$.

Flk1 (VEGF receptor 2) is one of the earliest marker of hemangioblast progenitors giving rise to vascular endothelial and hematopoietic lineages, smooth muscle cells and also to functional cardiomyocytes[50]. Morevover, Flk1+ progenitor cells were shown to improve cardiac function[149]. Interestingly, in mouse embryos, the Cited2 specific knockout in Nkx2.5-expressing cardiomyocytes resulted in a reduced coronary vascularization due to a reduction of *Vegf-*α expression in the heart[150]. Moreover, *Vegf-*α expression in the heart was showed to be directly controlled by Cited2[150]. Thus, we hypothesized that Cited2 might be promoting the differentiation towards hemangioblst progenitors and assessed the expression of *Flk1* and *Vegf-*α in cells overexpressing flag-CITED2 in comparison to the cells expressing the control vector (Figure 4.21). We observed in FC2 cultures an enhanced expression of *Flk1* and *Vegf-*α day 12 of differentiation. During embryonic development, the mouse *Flk1* gene which is produced in early lateral mesodermal progenitor cells at the streak stage and originate hematopoietic and vascular endothelial cell lineages[149]. At later stages, *Flk1* is expressed in endothelial cells, but its expression is lost in most hematopoietic cells[151]. The late enhancement of Flk1 and *Vegf-*α expression, at day 12 of differentiation, observed in ES cells overexpressing flag-CITED2, suggests that Cited2 might promote the differentiation and specification of cells derived from ES cells into endothelial cells.

4.3.4. Cardiac progenitor gene expression might be regulated by Cited2

4.3.4.1. Early cardiac lineages are enhanced in Cited2 gain-of-function cultures

Since an increment in mature cardiac gene markers was observed in cells overexpressing CITED2 (Figure 4.17), we wondered whether Cited2 could be involved at early stages of cardiac differentiation and examined the temporal gene expression pattern associated with early cardiogenesis using qRT-PCR to assess the expression levels of pro-cardiogenic genes.

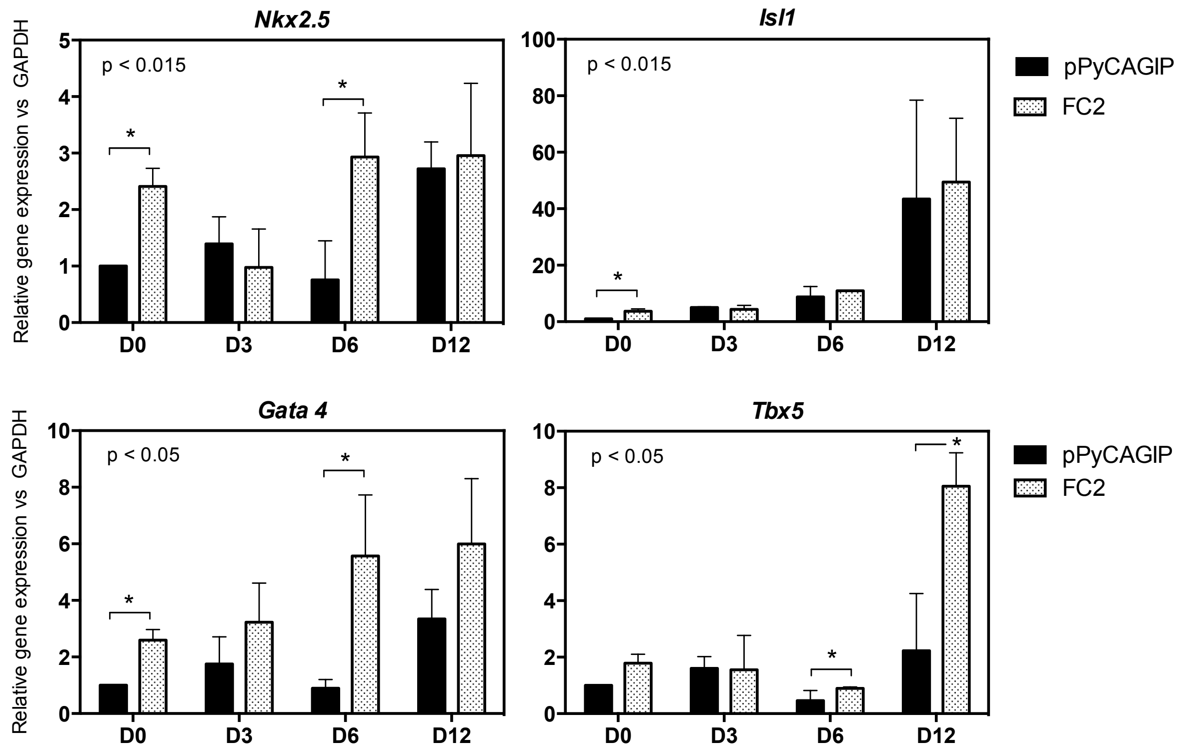


Figure 4.22. Cardiac progenitors markers gene expression during mESC in gain-of-function Cited2 experiments.

Relative gene expression levels of *Nkx2.5* and *Isl1* (upper panels), *Gata4* and *Tbx5* (lower panels) are shown. Control cultures (dark histogram) and FC2 cultures overexpressing Flag-Cited2 (dotted histogram) throughout differentiation. Gene expression was normalized against Gapdh expression and compared with pPyCAGIP gene expression at D0. The error bars correspond to SEM of triplicates and statistically significant values using a significance t-Student test were calculated, *p<0.05.

Genes encoding factors *Nkx2.5*, *GATA4*, *Tbx 5*, and other families were found to exert the functions of inductive signals during specification, patterning, and differentiation of the heart [152]. *Nkx2.5* is the only homeobox gene expressed throughout cardiogenesis and *Islet1* (Isl1) encodes a LIM homeodomain transcription factor that contributes to the embryonic heart, comprising cells of the outflow tract, right ventricle, both atria and the left ventricle [49, 153]. The earliest population of cardiac progenitors will give rise to cells forming the first heart field (FHF) that contributes to parts of the atrial chambers and the left ventricular region. The second cardiac progenitor population, will give rise to the second heart field (SHF), from which the out flow tract, the right ventricular region and the main parts of the atrial tissue are originated[48]. Both cardiac progenitors from FHF and SHF populations specifically express *Nkx2.5*, *Tbx5, Gata4*, *Mef2c* and *Isl1* transcription factors[62].

Surprisingly, in FC2 differentiating cultures, the expression of the inductors of cardiac cell commitment *Nkx2.5*, *Isl1* and *Gata4*, was elevated at the onset of differentiation, at day 0 when the cells are still in pluripotent conditions (Figures 4.22 and 4.23). The expression of *Gata4* and *Tbx5* was also elevated in FC2 transfected cells in comparison to control cells, at day 6 of differentiation (Figure 4.22). By day 12 of differentiation, only the expression of *Tbx5* was significantly higher in FC2 overexpressing cells (Figure 4.22). Interestingly, this result is in agreement with the decrease of Tbx5 expression observed in the cells derived from Cited2-knockout ES cells differentiation (Figure 4.7), indicating that the expression of TBx5 in late stages of ESC differentiation correlates directly with the expression of Cited2 and reinforcing the idea that Tbx5 might be a direct-target gene of Cited2. However, further experiments are required to demonstrate this hypothesis.

The increased gene expression of *Nkx2.5*, *Gata4*, *Isl1* and *Tbx5*, key regulators of numerous myocardial differentiation and developmental processes, observed in cells overexpressing Cited2 in mESC in the presence of LIF (at Day 0), suggests that Cited2 is either "priming" pluripotent mESC for the early activation of the cardiac cell program, or promoting the spontaneous differentiation of mESC towards cardiac progenitors when those cells are still cultured in medium allowing the maintenance of pluripotency

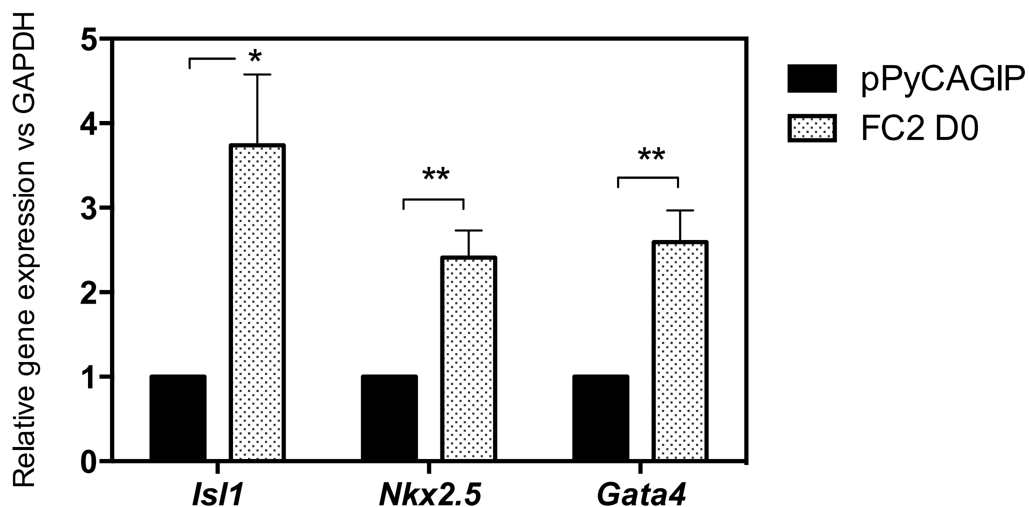


Figure 4.23. Primed mESCs by Cited2, towards cardiac progenitor specification, in gain-of-function Cited2 experiments.
Relative gene expression levels of *Isl1*, *Nkx2.5* and *Gata4* are shown. Control cultures (dark histogram) and FC2 cultures overexpressing Flag-CITED2 (dotted histogram) at stating stage during differentiation, day 0. Gene expression was normalized against Gapdh expression and compared with pPyCAGIP gene expression at day 0. The error bars correspond to SEM of triplicates, statistically significant values using a significance t-Student test were calculated, *p<0.015 and **p<0.05.

Taken together our results indicate that the overexpression of flag-CITED2 in mESCs promoted the specification of cardiac lineages at very early stages during normal differentiation.

4.3.4.2. Cited2 knockout impairs cardiac progenitor gene expression

Since Cited2 stimulated the gene expression of cardiac progenitors in pluripotent mESC overexpressing CITED2, also during mESC differentiation (Figure 4.22-23), we wondered whether cardiac progenitor gene expression could be altered in the absence of Cited2, because in Cited2-knockout experiments its depletion led to a decrease of *Brachyury* and *Mesp1* expression (Figure 4.11).

We hypothesize that there will be a discontinuance in cardiac progenitor emergence in Cited2^{4HT} differentiation cultures. Indeed, cardiac progenitors arise from cardiac mesoderm as two main and distinct cardiac progenitor cell populations that exist in early embryonic development and arise from ESC differentiation. On the other hand, we observed an increased expression of pro-cardiogenic genes, such as Nkx2.5, Gata4 and Isl1, in ESC overexpressing flag-CITED2 maintained in culture conditions for maintenance of pluripotency, indicating that Cited2 might facilitate and direct ESC fate to cardiac progenitors (Figure 4.23). Thus, in order to evaluate the effect of Cited2 depletion in cardiac progenitors differentiation, we analyzed the relative gene expression levels of cardiac progenitor markers, such as *Isl1*, *Nkx2.5*, *Gata4*, T*bx5* and *Mef2c*, at the stages when these progenitors were showed to emerge, in other words from day 2 to day 5 after the beginning of ES cell differentiation (Figure 4.24).

We compared the expression of the markers in Cited2^{Ethanol} and Cited2^{4HT} cell cultures, and observed no statistically significant differences in the expression levels of *Mef2c* and *Gata4* at days 2 and 3 of differentiation (Figure 4.24). On the other hand, *Isl1* expression, which marks the progenitors of the SHF, was slightly but significantly decreased at day 3 in Cited2^{4HT} cell cultures in comparison to control cells (Figure 4.24). Moreover, the reduction of Isl1 expression was even more pronounced in Cited2-depleted cells at day 5 (Figure 4.26).

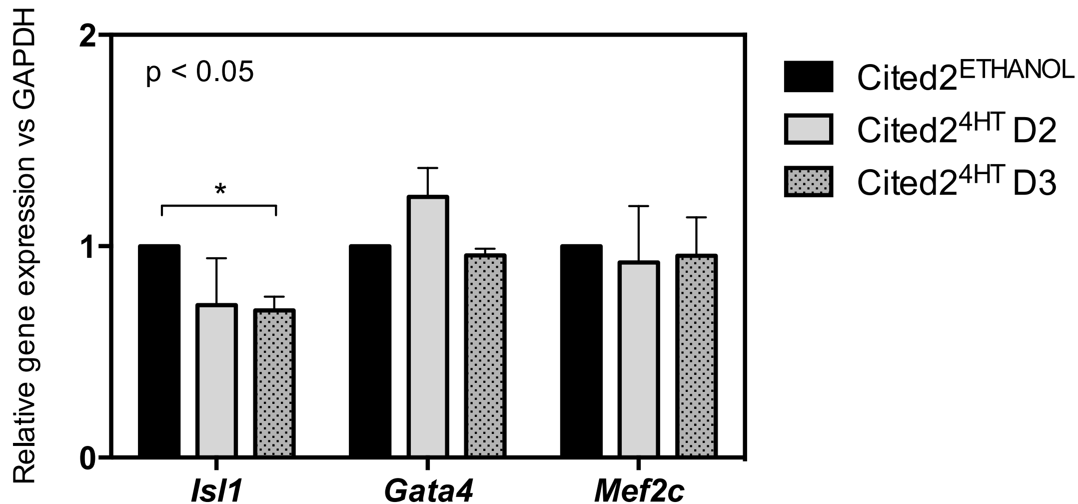


Figure 4.24. Relative gene expression levels for Isl1, Gata4 and Mef2c progenitor gene markers, at early differentiation stage, in Cited2$^{flox/flox}$ cultures treated with vehicle or 4HT, at day 0.
Differentiation day: 2 (dotted histogram) and 3 (grey histogram) of differentiation. The relative fold change expression was presented, comparing the values from each culture, Cited2^{4HT}, to its control, Cited2^{Ethanol} (black histogram). The error bars correspond to SEM of triplicates. The asterisk (*) correspond to statistically significant values using a significance t-Student test, $p<0.05$.

In addition, at day 2 of differentiation, the expression of neither *Nkx2.5* nor *Tbx5*, markers of the cardiac progenitor of the FHF, was detected in Cited2^{4HT} or control cell cultures, as expected since cardiac progenitors harboring these markers start arising after the second day of differentiation (like we normally observe in Figure 4.22). The expression of *Nkx2.5* and *Tbx5* was next determined in Cited2^{4HT} and control cell cultures (Figure 4.25). Surprisingly, no expression of *Nkx2.5* transcripts (n=4) was detected in mRNA extracts from Cited2^{4HT} cell cultures, while Nkx2.5 transcripts were present in control Cited2^{ETHANOL} cell cultures. Conversely, *Tbx5* transcripts were only detected in Cited2^{4HT} cells culture (Figure 4.25). The down-regulation of *Cited2* expression resulting from the 4HT treatment was also confirmed (Figure 4.25).

By day 5 of differentiation, in Cited2^{4HT} cell cultures, in addition to the strong reduction of Isl1 expression, the expression of Mef2c, Gata4 and Tbx5 was slightly but significantly decreased, while the expression of *Mesp1* or *Nkx2.5* transcripts was not significantly altered in comparison to the equivalent control cell cultures (Figure 4.26).

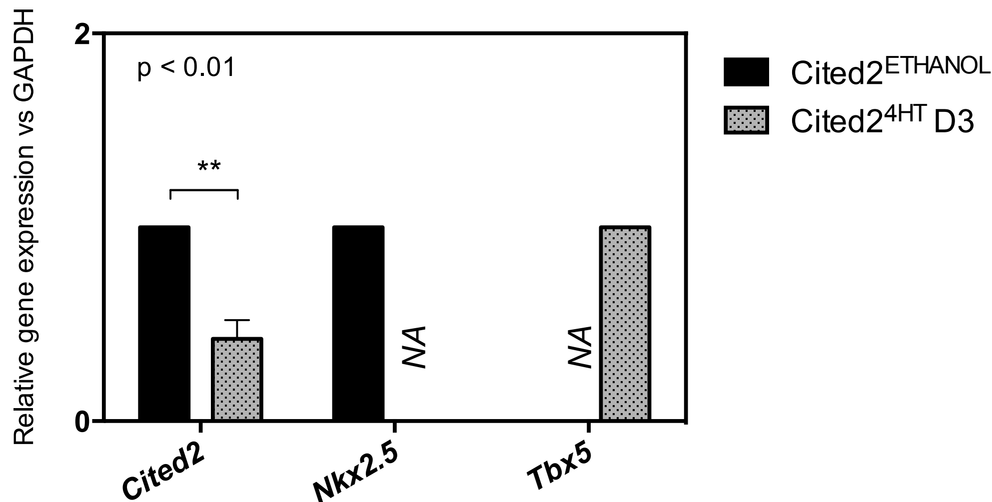


Figure 4.25. Relative gene expression levels for Cited2, Nkx2.5 and Tbx5 during specification stage, from Cited2$^{flox/flox}$ cultures treated with vehicle or 4HT, at day 0.
The relative fold change expression was presented, comparing the values from each culture, Cited2^{4HT} (dotted histogram) to its control, Cited2^{Ethanol} (black histogram). NA: no amplification. The error bars correspond to SEM of triplicates. Statistically significant values were calculated using a significance t-Student test, **p<0.01.

Overall, these data suggests that Cited2 is required for the efficient emergence of mesodermal and cardiac mesoderm cells since we have showed that Cited2-depletion at the beginning of the differentiation results in the marked decrease of *Brachyury* and *Mesp1* (Figure 4.11). The cardiac progenitors differentiation is also affected by Cited2-depletion, as indicated by the decreased expression of Isl1 at days 3 and 5, the decreased expression of Nkx2.5 at day 3, and Gata4 at day 5 (Figures 4.24, 4.25 and 4.26). This latter observation was expected since cardiac progenitors derive in a straight line from the differentiation of cardiac mesoderm. Interestingly though, Cited2 mostly affected the expression of *Isl1* at later stages (day 3 to day 5), suggesting that Cited2 is more essential for differentiation of progenitors of the SHF than for the progenitors of the FHF. The data obtained is in agreement with previous reports that have showed that in embryonic development Cited2-knockout affects most of the heart structures derived from the SHF[76].

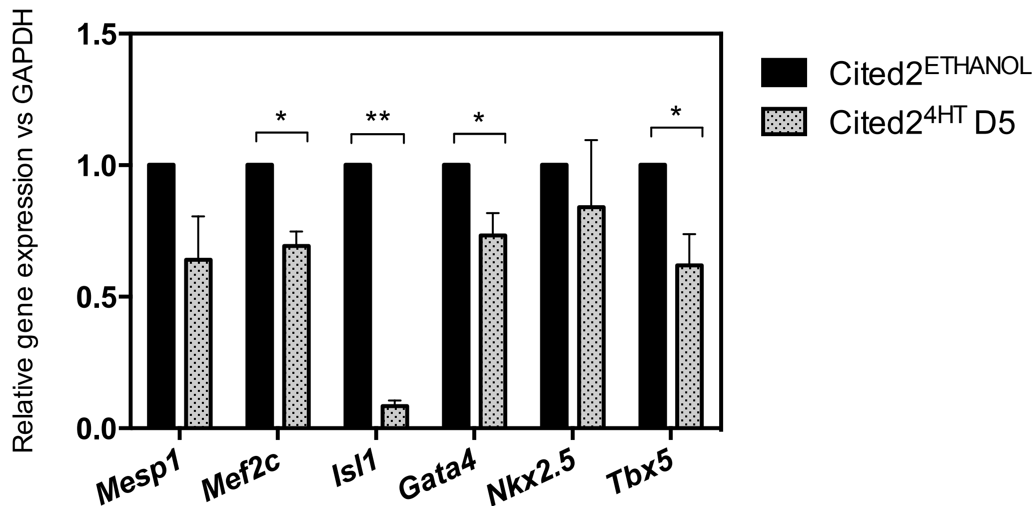


Figure 4.26. Cardiac progenitor relative gene expression levels at day 5 of differentiation, in Cited2$^{flox/flox}$ cultures treated with vehicle or 4HT, at day 0.
The relative fold change expression was presented, comparing the values from each culture, Cited2^{4HT}, to its control, Cited2^{Ethanol} (black histogram). The error bars correspond to SEM of triplicates. Significant values using a significance t-Student test were calculated, *$p<0.05$ and **$p<0.01$.

4.4. Second heart field progenitors

4.4.1. Cited2 expression is present in mesodermal precursors

The data that we have obtained studying Cited2 knockout ESC differentiation have revealed that Cited2 is required for the efficient emergence of mesodermal, cardiac mesoderm cells and strikingly affected the expression of the SHF cardiac progenitors marker Isl1. In addition, there is evidence published indicating that *Cited2* is co-express with pro-cardiogenic transcription factors such as *Gata4*, *Isl1* and *Mef2c* when mesodermal commitment occurs during mouse heart development [154]. Moreover, the overexpression of flagCITED2 in ESC also enhanced the expression of pro-cardiogenic markers, such as *Isl1*, *Gata4* and *Nkx2.5* in cells maintained in undifferentiating culture conditions. Thus, we hypothesized that Cited2 itself might play an active and direct role in the generation of cardiac progenitors.

To establish whether, Cited2 expression was associated with cardiac progenitors of the FHF and SHF, we used the Apple D2 mouse ES cell line (AD2). AD2 cell line harbours the Green Fluorescent Protein (GFP) gene under the control of *Nkx2.5* promoter and the dsRed reporter gene under the control of a regulatory element of the *Mef2c* gene[115]. The differentiation of AD2 ES cells will trigger the expression of: i) GFP in cells expressing Nkx2.5 and will consequently mark FHF progenitors and cells derived

from these progenitors; ii) dsRed in cells initially expressing Isl1 which acts positively on the Mef2c regulatory element driving the expression of dsRed, and therefore will mark cardiac progenitors of the SHF; and iii) expression of both GFP and dsRed in cells (double^{++}) which are a multipotent mesodermal progenitors population[115].

AD2 mES cells differentiation was induced by embryoid body (EB) formation using the hanging drop method as described above. After 6 days of differentiation, double transgenic cells were sorted by FACS to obtain 4 populations based on the GFP and dsRed expression: double negative (GFP^{-} and dsRed^{-}), double positive (GFP^{+} and dsRed^{+}), only red (dsRed^{+-}) and only green (GFP^{-+}) populations (Figure 4.27A). Cells that expressed both fluorescent proteins will be referred hereafter as multipotent mesodermal progenitors (MMP), red positive cells as second heart field progenitors (SHF) and green positive cells as first heart field progenitors (FHF)[115].

The double positive cells are mainly multipotent mesodermal progenitors that will contribute to populate the heart when this one is formed. The Red^{+} and Green^{+} population of cardiac progenitors are expressed as soon as the heart starts to be formed [115].

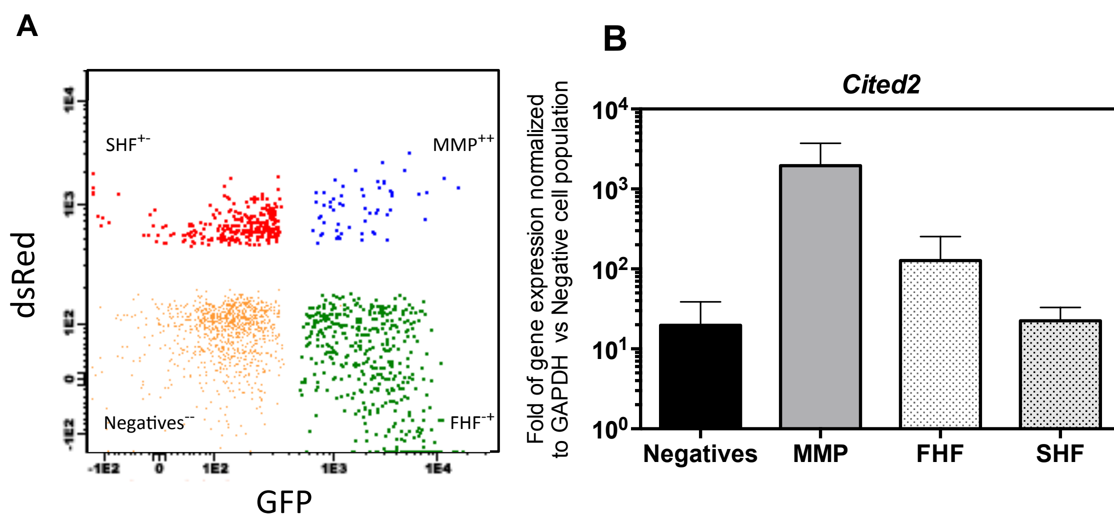


Figure 4.27. Cited2 transcripts in cardiac progenitors.
(A) Representative flow cytometry plots of double transgenic differentiate cells at day 6, showing four populations of cells: double negatives (negatives$^{-/-}$), double positives$^{+/+}$ (MMP$^{+/+}$), GFP positives (FHF$^{+/-}$) and dsRed positives (SHF$^{-/+}$).
(B) The populations were used to prepare total RNA and perform an qRT-PCR. Expression of *Cited2* was normalized to *GAPDH* expression and reported as a fold of expression of *Cited2* expression in negative cell population. *Cited2* expression in differentiated culture cells at day 6. The error bars correspond to SEM of *n*=4.

We isolated the total RNA from the four populations and analysed the Cited2 transcripts expression by qRT-PCR (Figure 4.27B). We observed that Cited2 transcripts

were enriched in all cardiac cell populations when compared to double negative cells (Figure 4.27B), with higher expression levels in MMP cells. Although *Cited2* gene expression levels do not present statistical significance between the populations, which might be related with the stochastic *in vitro* differentiation, this result indicates that *Cited2* is co-expressed within cardiac progenitors.

In all of the experiments performed (*n*=3) the *Nkx2.5* transcripts failed to be detected in multipotent mesodermal progenitors (double^{++}) population (Figure 4.28) but they were present in FHF (GFP^{+}) and SHF (dsRed^{+}) population (Figure 4.29). This result is intriguing, since *GFP* gene is under the control of the *Nkx2.5* promoter and cells were sorted based on the GFP expression (Figure 4.27B). Possible explanations might be that GFP protein is more stable than the *Nkx2.5* transcripts or *Nkx2.5* transcripts were systematically degraded in this cell population during the extraction. Because *Gata4* is expressed in the major lineages of the developing and mature heart[155], we assessed its expression in all four cell populations. The *Gata4* expression profile that we observed (Figure 4.28) was in agreement with previous reports indicating that MMP and FHF populations presented a higher expression of Gata4 than SHF populations[115].

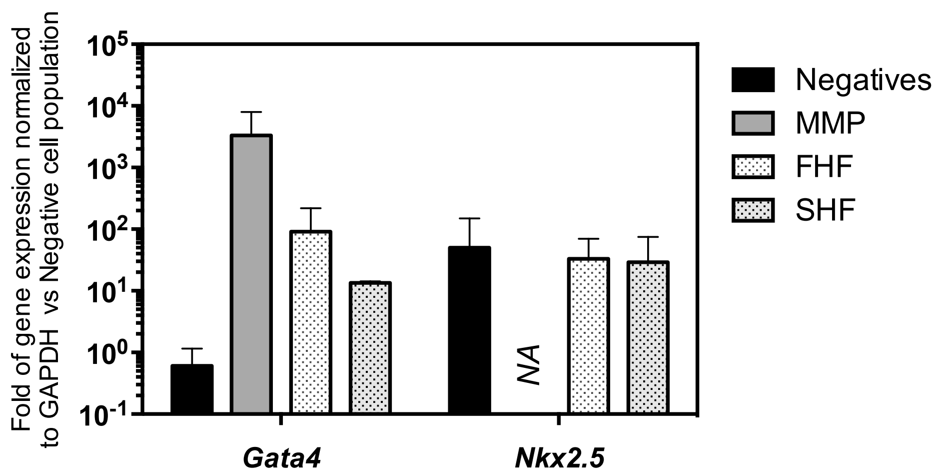


Figure 4.28. Gene expression of earliest cardiac transcription factors to be expressed during development, Gata4 and Nkx2.5.
FACS Sorted cell population of: Negatives$^{-/-}$ (black histogram), MMP$^{+/+}$ (gray histogram), FHF^{+}/GFP^{+} (light dotted histogram) and SHF^{+}/dsRed^{+} (dark dotted histogram). Gene expression was normalized to *GAPDH* expression and reported as a fold gene expression compared to negative cell population. NA= no amplified. Samples obtained at day 6 of differentiation. The error bars correspond to SEM of *n*=4.

The expression of Isl1 was enriched in SHF progenitors as expected (Figure 4.29, right panel). We found some discrepancy between our result compared to previously

reported using the same mES cells AD2 line, in which they observed high expression of Isl1 and Mef2c transcripts [115]. This discrepancy could be due to the inherent differences between lab conditions (i.e. bovine serum batch) that affect SHF positive population.

On the other hand, FHF population (green^{+}) presented expression of gene markers *Tbx5* and *Nkx2*.5 (Figure 4.29), at similar levels to those previously reported [115].

To summarize, *Cited2* expression was associated with essential cardiogenic genes. *Cited2 expression was* mainly enriched in the MMP and SHF progenitor cells populations that will give rise either the heart structure (MMP) or in cells that populate outflow tract, right ventricle and the two atria (SHF).

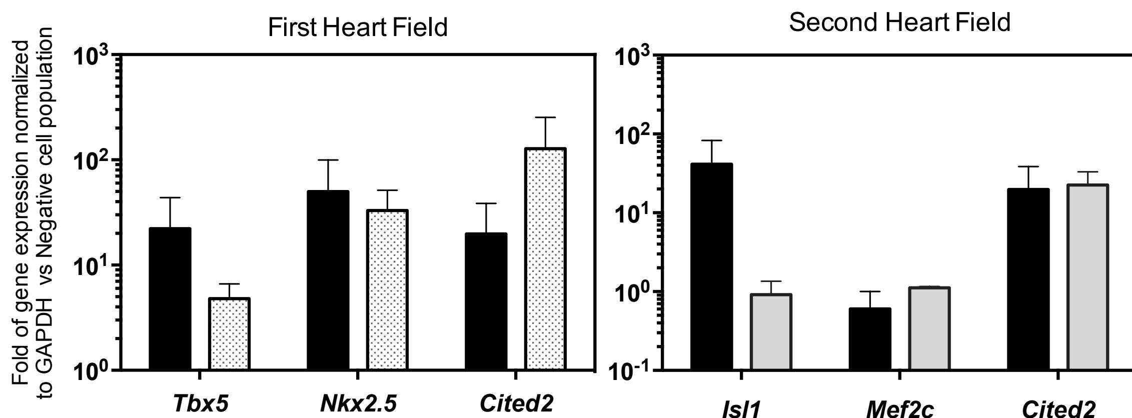


Figure 4.29. Gene expression of cardiac progenitors in first and second heart fields progenitors.
Left panel, gene expression of first heart field related genes; Right panel, relative gene expression of second heart field related genes, from FACS Sorted cell population of: Negatives$^{-/-}$ (black histogram), MMP$^{+/+}$ (gray histogram), FHF^{+}/GFP^{+} (light dotted histogram) and SHF^{+}/dsRed^{+} (dark dotted histogram). Gene expression was normalized to *GAPDH* expression and reported as a fold of gene expression compared to negative cell population. Samples obtained at day 6 of differentiation. The error bars correspond to SEM of *n*=4.

4.4.2. Cited2 gain-of-function in AD2 embryonic stem cell line

Data presented in the previous sections suggested that *Cited2* ectopic expression in mESC primed them towards cardiac progenitors cells, at least it led to an increase of the expression of genes expressed in cardiac progenitors, such as *Isl1*, *Gata4* and *Nkx2.5*. Taking further this hypothesis, we tested whether the ectopic expression of *Cited2* in the double transgenic AD2 mESC line could influence the normal rise of cardiac progenitor cells. A transiently transfection of these cells with the plasmid

overexpressing CITED2 (denominated AD2^{FC2} for simplicity) or the control empty vector (denominated AD2^{pPyCAGIP} for simplicity), was performed two days before the onset of differentiation. The emergence of cardiac progenitor cells was monitored every day after the beginning of differentiation using a FACS analyser, from day 0 up to day 7. The time course of fluorescence expression for MMP, SHF and FHF cells was determined and compared between AD2^{FC2} and AD2^{pPyCAGIP} cells (Figure 4.30). A consistent increase in fluorescence levels was detected in cell populations (MMP, SHF and FHF cells) in AD2^{FC2} cultures at day 6 but failed to reach statistical significance (Figure 4.30).

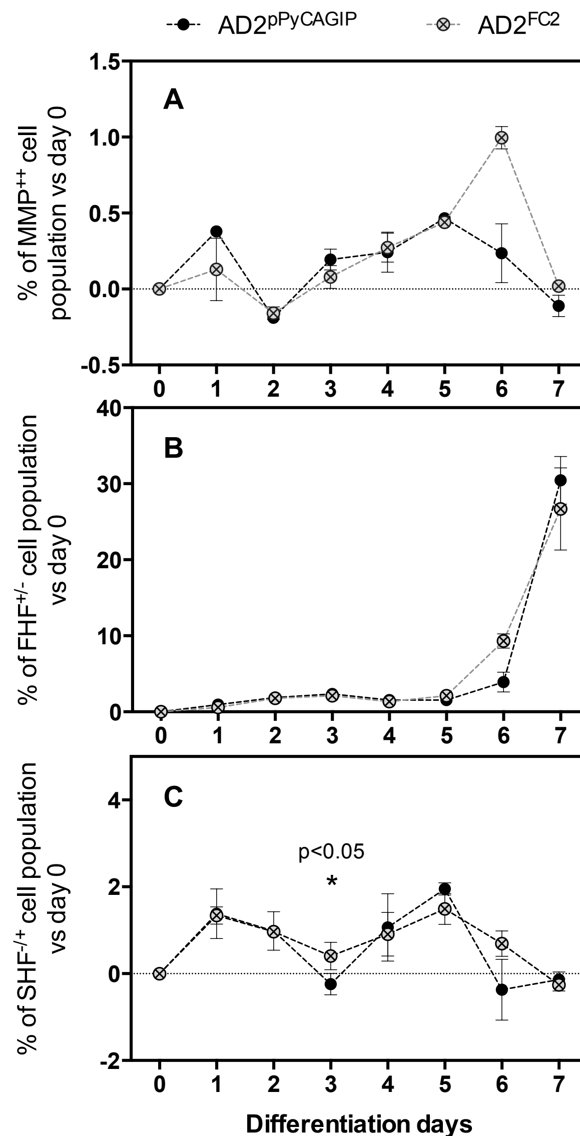


Figure 4.30. Influence of Cited2 overexpression on the emerging cardiac progenitors from mESCs.
Panels represent cell populations of: (A) Multipotent mesodermal progenitors (MMP^{++}); (B) SHF progenitors (SHF$^{-/+}$), and (C) FHF progenitors (FHF$^{+/-}$). Control AD2^{pPyCAGIP} (-●-) and AD2^{FC2} (-⊗-) cells, differentiation days (D) are indicated. Values were calculated from % of fluorescence at given point normalized against day 0 fluorescence. The error bars correspond to SEM of triplicates, statistically significant values using a significance t-Student test were calculated, *$p<0.05$.

Overall, Cited2 overexpression did not affect the normal expression of GFP and dsRed in all population of progenitors, apart at day 3 of differentiation of SHF cells where the $AD2^{FC2}$ cells dsRed fluorescence detection was significantly increased compared to control $AD2^{pPyCAGIP}$ cells.

Taken together, the results showed that *Cited2* is expressed in cardiac progenitors, the transient overexpression of CITED2 might modestly promote the emergence of SHF progenitors at day 3 of differentiation.

4.4.3. Isolation of second heart field progenitors from AD2 differentiated cultures

In order to understand the role of Cited2 during the cardiac progenitor stage, specifically in SHF cells, we isolated dsRed positives cells differentiating from the double transgenic AD2 mES cell line by FACS (Figure 4.31.1).

Several reports have showed that Wnt/β-catenin signaling pathway stimulates the maintenance and proliferation of cardiac progenitors of the secondary heart field in culture[61, 63, 156, 157]. The isolated $dsRed^{+}$ cells, hereafter referred as second heart field progenitors (SHF^{p}), were maintained in culture for 10 cell passages with supplementation of 2.5μM of an inhibitor of glycogen synthase kinase-3 (GSK-3) that activates the β-catenin, (Figure 4.31.2).

The expression of dsRed was detected in the isolated and amplified cells maintained in culture in the presence of the GSK-3 inhibitor for to 10 passages (Figure 4.31.2). However, to characterize further these cells expressing dsRed (SHF^{p} cultures), we assessed the expression levels of *Isl1* and its target gene, *Mef2c*, which is an early marker for SHF^{p} [115, 158], along with *Mesp1* and *Cited2* expression (Figure 4.32). The sustained expression of *Mef2c* in SHF^{p} cultures is in agreement with the detection of dsRed expression in these cells, since the dsRed gene is under the control of the Me2fc enhancer. At early passages (CP-3), the expression of Isl1 and Mesp1 was higher than at later passages (CP-7 and CP-9) in isolated SHF^{p} cells, and conversely the expression of Cited2 and Mef2c was increased at later passages (Figure 4.32).

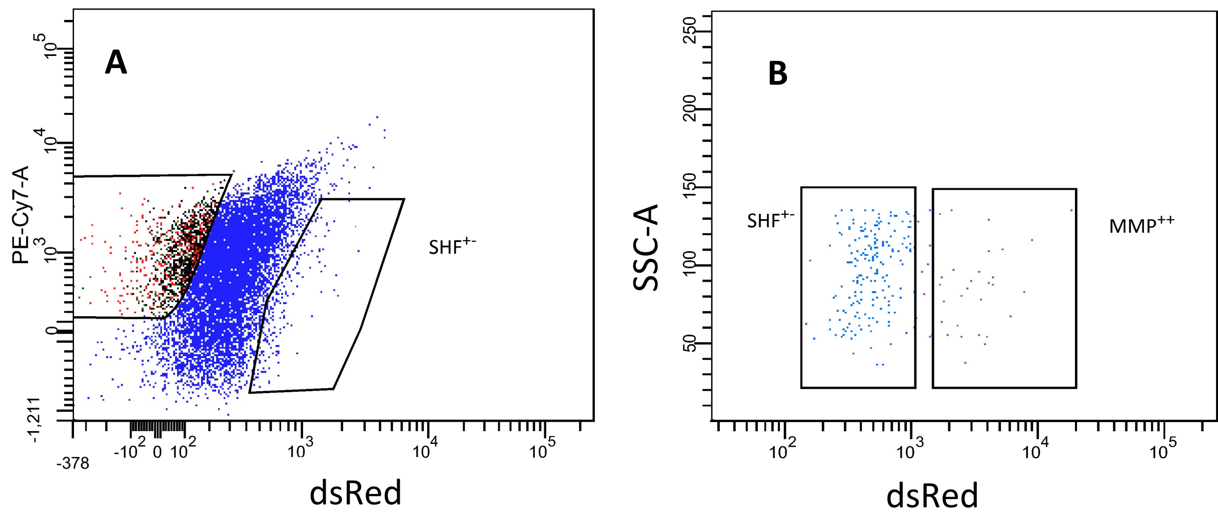
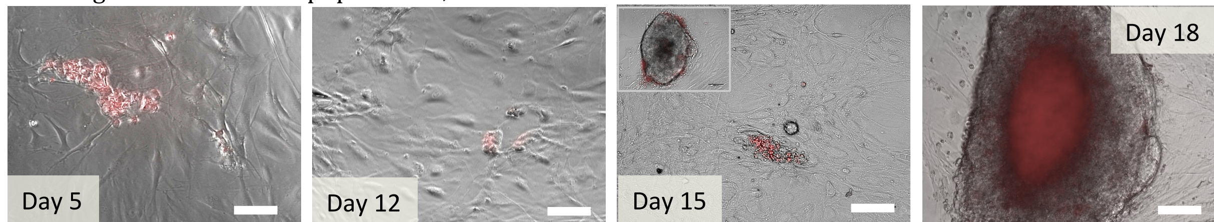


Figure 4.31.1. Isolation of SHF cardiac progenitors by FACS.
(A) Representative flow cytometry plot of differentiated AD2 cell line compensation dye in fluorescen *y*-axis and dsRed fluorescence in *x*-axis. (B) The cells positives for dsRed fluorescence (*x*-axis) and a Showing SHF+- and MMP++ populations, from where SHF+- cells were sorted.



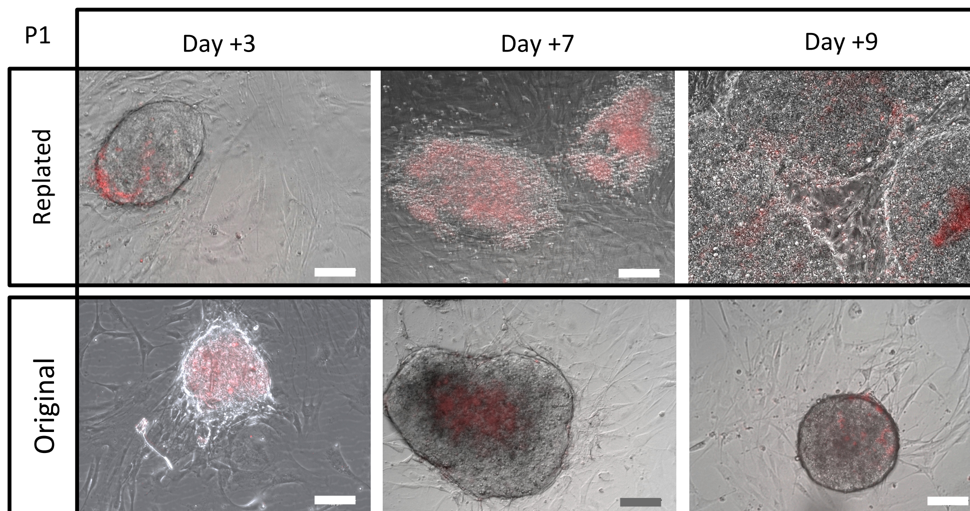


Figure 4.31.2 . SHF progenitors isolated from ES cells EBs at day 4 of differentiation and cultured in vitro.
Representative microphotography of SHFp cultures maintained with GSK3 inhibitor in Mefs, bar represents 100μm; cells were isolated from AD2 differentiated EB cultures at day 4 by FACS sorting, taking advance of their dsRed fluorescence when cells undergo trough cardiac differentiation program; the dsRed is under control of the second heart field enhancer. Maintenance of SHFp was done by supplement medium culture with BIO (5mM), LIF (0.125 *ng*/μl) and using inactivated MEF. Besides the dsRed+ cells are around the less than 1% during the AD2 differentiation we succeed in maintain them in culture (upper panel). By day 18th after isolation, the SHFp with strong signal were selected (3 colonies) and replated (lower panel). Original cultures were maintained as well. Nine day after the fist cell passage, the morphology of cultures resembles the mESC cultures. At this point the second cell passage was done. Cultures were maintained until cell passage number 9.

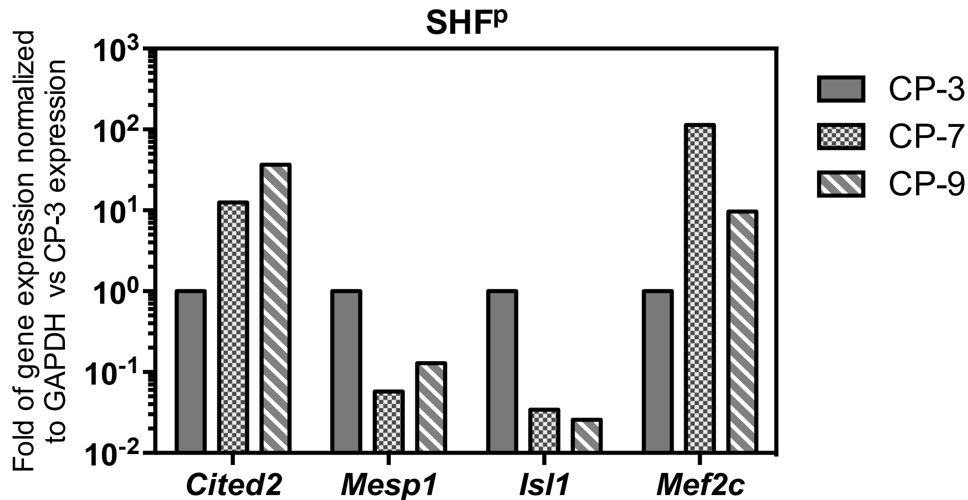


Figure 4.32. Cardiac gene expression in SHF^p cultures at different cell passages.
Cited2, *Mesp1*, *Isl1* and *Mef2c* gene markers were analyzed at different cell passages (CP): 3 (gray histogram), 7 (dotted histogram) and 9 (stripped histogram). Gene expression was normalized against Gapdh and compared to CP3 gene expression setting arbitrarily to 1, for each gene; *n*=1.

The expression of mesendodermal/endodermal (*Brachyury*, *FoxA2, AFP*), pro-cardiogenic (αMHC) and pluripotency (*Nanog*, *Oct4, Sox2*) genes in SHF^p cell as at passage 3 is consistent with the early cardiac progenitor nature of these cells (Figure 4.33). The lack of detection of *αMHC* and *Nestin* transcripts expression, and the small levels of *AFP* expression indicated that these cells are not differentiated. The expression of β-III-tubulin detected in our samples might be from an aberrant expression of these progenitors, or from a contamination with neural cell types, such as motor neurons which differentiation is promoted by Isl1.

Intriguingly, the cells seemed to self-renew, to proliferate and to sustain dsRed expression suggesting that these cells were not differentiating, at least not all of the cells. Furthermore, we did not observe any contractile areas during SHF^p cultures. Therefore, if the cells were differentiating, they were not differentiating into cardiomyocytes. We hypothesized that the early progenitors observed at passage 3 which expressed *Isl1* and *Mesp1* were switching to different progenitor cells from passage 3 to passage 7 that curiously expressed higher levels of Cited2 and Mef2c transcripts and less *Isl1* and *Mesp1* transcripts. Noticeably, at passage 9 the expression of *Cited2*, *Mef2c*, *Isl1* and *Mesp1* was comparable to their expression at passage 7, indicating that the expression of the tested genes reached steady-state levels by the 7th passage, and suggesting that the cells expressing dsRed and higher levels of Cited2 and

Mef2c were stable in our culture conditions. Moreover, the fact that these cells at passage 7 were able to differentiate and contractile foci (Figure 4.35), reinforces the hypothesis that the SHF^p cells expressing dsRed, and high levels of Cited2 and Mef2c are cardiac progenitors. Although, we have followed the expression of the Isl1, Cited2, Mef2c and Mesp1 markers in SHF^p cells expressing dsRed over 9 passages, this experiment was only successfully performed once due to the scarce number of dsRed progenitors that are derived from AD2 cells. Thus, before drawing any further conclusion, we will need to confirm these data.

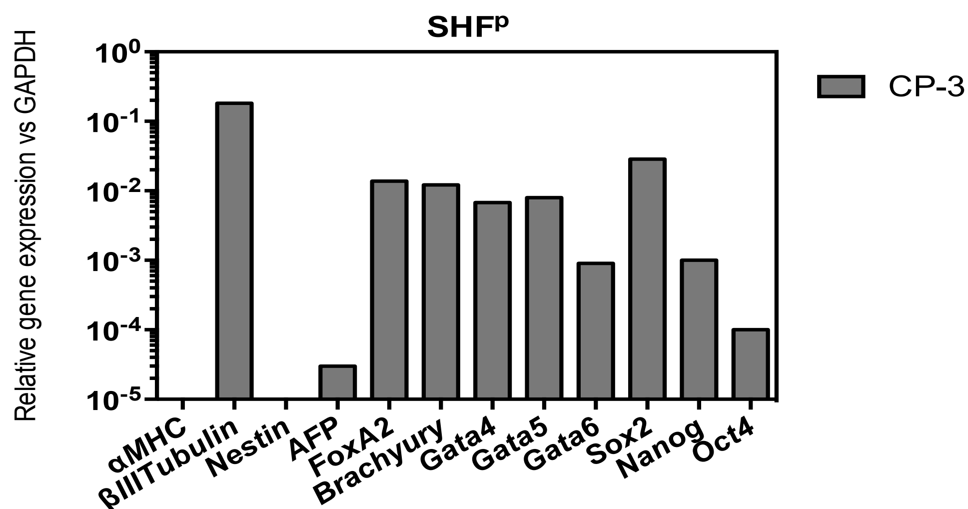


Figure 4.33. Expression profiling SHF^p cultures at cell passage 3.
Specific gene markers were analyzed at cell passages (CP) 3 (gray histograms); mature cells: *αMHC*, *β–III–Tubulin*, *Nestin*, *AFP*; mesoendodermal intermediates: *FoxA2*, *Brachyury*, *Gata4-6*; pluripotency: *Sox2*, *Nanog*, *Oct4*. Gene expression was normalized to *GAPDH* expression. *n*=1.

4.4.3.1.1. Cited2 overexpression in Second Heart Field progenitors block cardiac cell commitment

After characterization of SHF progenitors, we perform a Cited2 overexpression or Cited2 knockdown in those cells, in order to determine whether Cited2 was only important for the induction of cardiac mesoderm or if was also important for maintaining the progenitor status of the cells.

For gain-of-function cultures in SHF^p cells, we transfected a pCagip-FlagCited2 plasmid, and its control pPyCAGIP, as described in the above sections. Cited2 knockdown was performed using a plasmid, expressing a double-stranded RNA (dbRNA) targeting Cited2 messenger (Cited2-knockdown) and a control vector expressing an irrelevant dbRNA. Isolated SHF^p cells maintained in culture until passage

number 7 were used. Two days post-transfection, cells were used for EB formation and at day 5 after EB formation the expression of relevant genes was analyzed. The differentiation cultures were kept until day 12 after EB formation.

At day 5 of differentiation, we noticed lower *Mef2c* transcript levels in SHF^{p} cells that overexpress Cited2, Isl1 expression levels present similar behavior but still not conclusive results; whereas knocking-down Cited2 seem to affect *Isl1* transcripts (figure 4.34).

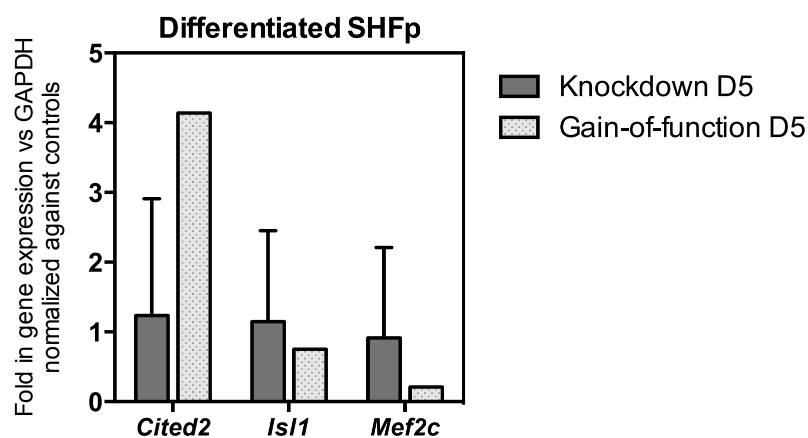


Figure 4.34. Cited2 and SHF gene markers in differentiated SHF^{p} cultures.
Cited2, *Isl1* and *Mef2c* genes were analyzed in Cited2-knockdown (gray histogram) or Cited2-gain-of-function (dotted histogram); samples were obtained at day 5 of differentiation. Gene expression was compared against to *Gapdh* expression and normalized vs gene expression in controls. The error bars correspond to SEM with duplicates.

By day 8 of differentiation presented more contractile areas when Cited2 was knockdown from SHF^{p} (figure 4.35). Although these results are very preliminary data, they might suggest a relationship in Cited2 gene expression and cardiac progenitor renewal. One possible explanation for this is that levels of Cited2 protein were inhibiting or delaying terminal cardiomyocyte differentiation by keeping the SHF^{p} in a progenitor-like state.

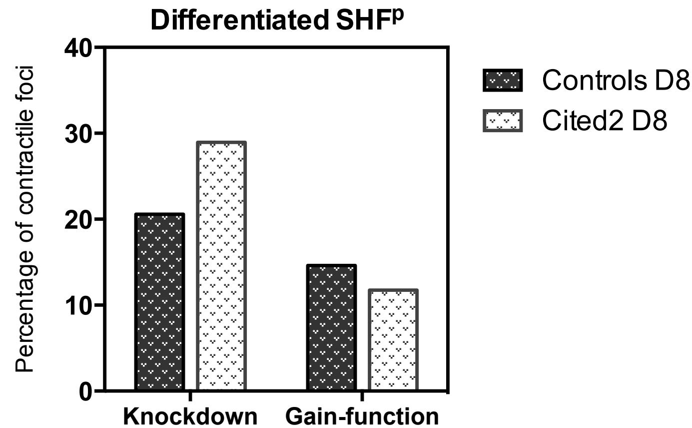


Figure 4.35. Consequences of Cited2 dosage over contractile foci from SHFp cardiac cell differentiation.
Contractile foci were counted at day 8 and normalized against the total number of colonies (day 12); the dotted histograms represent SHFp cultures subjected either to Cited2-knockdown (dark) or Cited2-gain-of-function (light); *n*=1.

4.5. Gata4 and Cited2 overexpression in embryonic stem cells

We have also shown that Cited2 and Gata4 interact in vitro, thus we will be confirm this interaction in vivo, and the functional effects of this interaction on genes involved in the cardiac differentiation program that are targets of Gata4, such as Nkx2.5, Isl1 and Mef2c. Here, we have cloned the Gata4-ORF into pPyCAGIP plasmid for expression in mES cells.

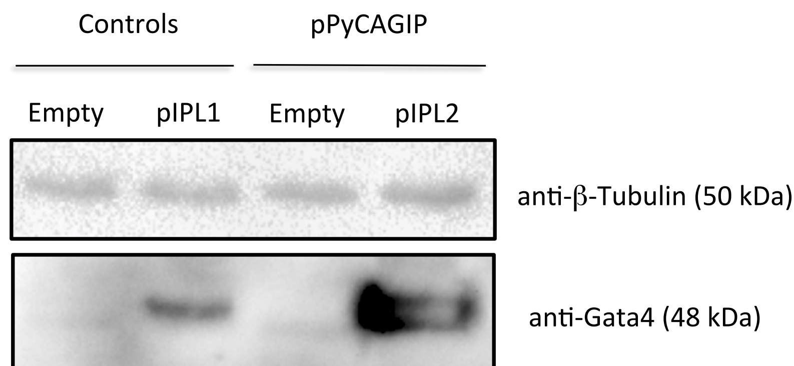


Figure 4.36. Gata4 protein detection in HEK293T cells.
Hek293T cells were transiently transfected with pBSSK or pPyCAGIP derivatives vectors. Empty, controls vector; pIPL1, pBSSK-GATA4; pIPL2, pPyCAGIP-GATA4. Antibodies dilutions used as follows, anti-β-Tubulin, 50kDa, 1:1000 and anti-Gata4, 48 kDa, 1:2000.

Its construction involved the construction of an intermediary plasmid to get the right restriction sites (XhoI-NotI) to clone in to pPyCAGIP vector. This intermediary vector (pIPL1) was obtained by recombination (EcoRI) of a PCR amplification product harboring the GATA4-ORF between restriction sites, using pBluescript II SK (pBSSK) as

backbone. Then vectors were transfected in HEK293T cells for further protein analysis (figure 4.36).

4.5.1. Gata4 and Cited2 gain-of-function

Since ours results pointed out to a direct effect of Cited2 expression towards a mesodermal commitment during mES cell differentiation, the previous indication of a physical interaction between Cited2 and Gata4, and that Cited2 expression has been reported to overlap with the expression of Gata4, Gata5 and Gata6[154, 159] during the primitive stage in mouse development, we decided to test whether CITED2 and Gata4 act together to promote cardiac cell commitment, by gain-of-function experiments at the beginning of ESC differentiation.

We used E14/T mES cell lines, either stably carrying a control vector (pPyCAGIP-GFP) or one plasmid constitutively expressing CITED2 (pPyCAGIP-FlagCITED2, FC2), and super-transfected with the same quantities of control and overexpressing Gata4 vectors (pPyCAGIP and pPyCAGIP-GATA4). Two days after transfection, cells were collected and plated for EB differentiation and, at day 12 of differentiation the forming colonies and beating foci were counted.

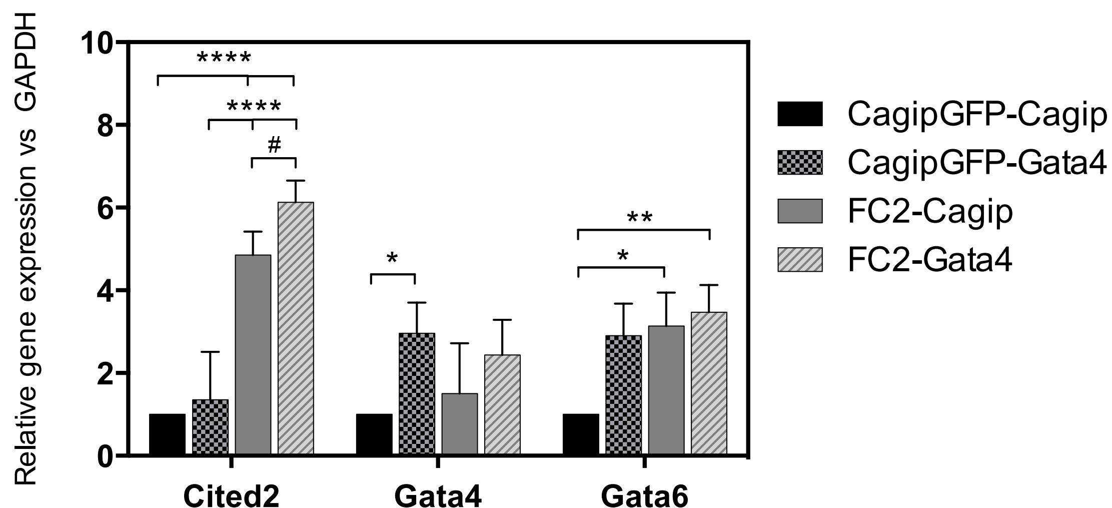


Figure 4.37. Cited2, Gata4 and Gata6 gene expression in ESC differentiated cultures at day 12, in gain-of-CITED2-Gata4-function experiments.
Relative gene expression in control cultures (CagipGFP-Cagip/ black histogram); cultures overexpressing Gata4, (CagipGFP-Gata4/ dark dotted hisogram); cultures overexpressing CITED2 (FC2/ gray histogram); and cultures overexpressing both CITED2 and Gata4 (FC2-Gata4 / light histogram). Normalized against Gapdh and compared vs day CagipGFP-Cagip that was arbitrarily set to 1. Transfection was carried with 0.5µg of total DNA. Statistical significance was analyzed by ANOVA with post-hoc Dunnett's test (*,#P < 0.05, **P <0.0018, ****P < 0.0001, n = 3) Mean ± s.e.m., n = 3 per group.

The results in Figure 4.37 show that levels in Cited2 transcript are not altered in control cultures. Moreover, the overexpression of Gata4 (CagipGFP-Gata4) did not alter significantly Cited2 transcript levels when compared with control (CagipGFP-Cagip). As expected, in cultures that overexpressed CITED2, the levels of Cited2 transcripts were increased in both culture conditions FC2-Cagip and FC2-Gata4, although a significant slightly higher expression was found in FC2-Gata4 cultures.

The expression of Gata6 is tightly associated with Gata4 expression[159]. The expression of both Gata4 and Gata6 transcripts in cultures that constitutively express CITED2 (FC2-Cagip) appears to be different when comparing with controls (Figure 4.37). This observation correlates with our previous results where Gata4 transcripts are increased (≈2-3 fold) when CITED2 was overexpressed in mESC (Figure 4.22 and 4.23). Although Gata4 expression levels were approximately similar in all cultures when compared with control, only statistically differences was found in cultures were Gata4 was overexpressed (GagipGFP-Gata4) and, no high expression level of Gata4 was found in cultures overexpressing both proteins (FC2-Gata4), as we were hypothesizing, However, this late might correlate with the observation in a degradation band related to Gata4 protein, when both proteins, CITED2 and Gata4, are overexpressed (Figure 4.38).

Gata4 protein was detected by immunoblotting at the beginning of differentiation (Figure 4.38), even in control cultures as reported before, that a band corresponding to GATA4 protein was detected in undifferentiated ESC as well as in blastocyst [160].

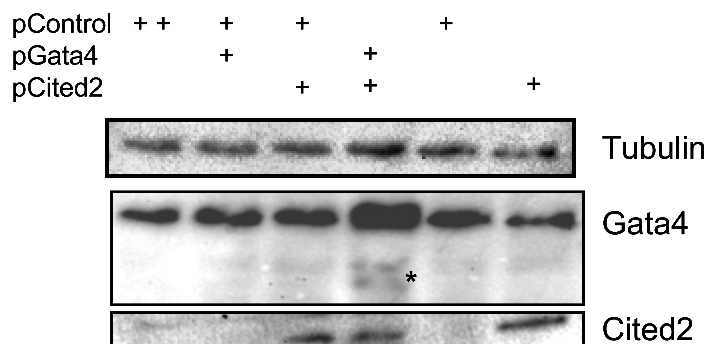


Figure 4.38. Protein detection of Gata4, CITED2 and b-Tubulin.
mESC cultures were transiently transfected either with a control vector, pCAGIP-GFP (pControl); a vector overexpressing Gata4, pPyCAGIP-Gata4 (pGata4) or a vector overexpressing CITED2 (pCited2). Dilutions: Anti-β-Tubulin, 50kDa, 1:1000; Anti-Gata4, 48 kDa, 1:200; Anti-Cited2, 28 kDa, 1:500. (*) Asterisk marks an additional band at 15-20 kDa that correspond to cleavage of GATA4.

An additional band at 15-20 kDa was detected in cultures expressing both CITED2 and Gata4 (Figure 4.38). This result suggests that CITED2 might be involved in GATA4 degradation or stabilization, although protein detection at late stages needs to be determined.

Since Gata4 is capable of specifying endoderm cell fates, we analyze the expression gene markers, which have been associated with Gata4, like FoxA2 that is expressed in the visceral endoderm, Flk1 expressed in mesoendodermal cells and, also for AFP that marks cells that follows a liver fate[14, 147]. In Figure 4.39, the results show that gene expression of FoxA2, AFP and Flk1 in cultures overexpressing Gata4 where up regulated, in agreement with previous reports. This also occured when Cited2 was constitutively expressed. Indeed, the expressions of those gene markers was up regulated, although in the case of FoxA2 the difference was not significant when compared to cultures transfected with control vectors. Surprisingly, Flk1 was up regulated either in presence of Gata4 or CITED2 alone (≈4 fold), but was a decreased when Gata4 and CITED2 were overexpressed simultaneously (Figure 4.39).

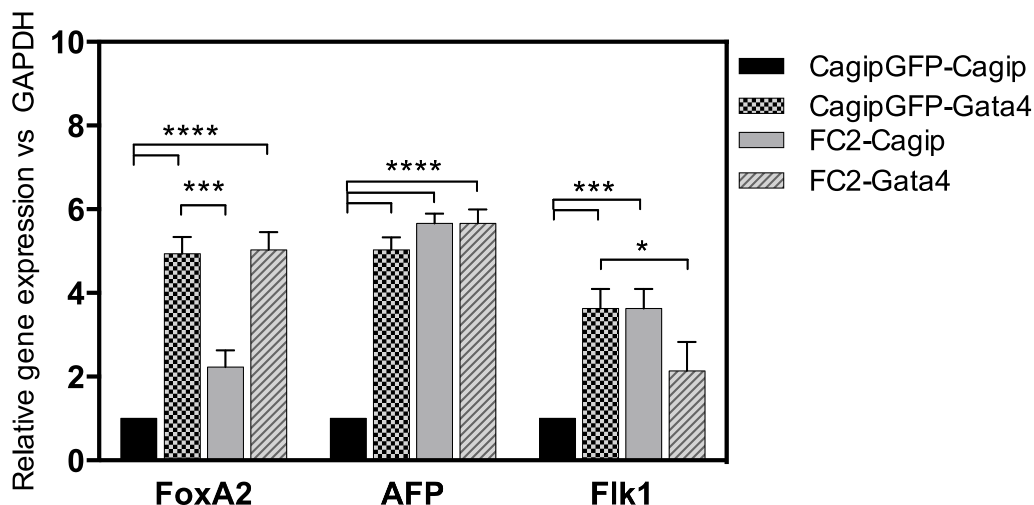


Figure 4.39. Endonderm gene markers in ESC differentiated cultures at day 12, in gain-of-CITED2-Gata4-function experiments.
Relative gene expression in control cultures (CagipGFP-Cagip/ black histogram); cultures overexpressing GATA4, (CagipGFP-Gata4/ dark dotted hisogram); cultures overexpressing CITED2 (FC2/ gray histogram); and cultures overexpressing both CITED2 and Gata4 (FC2-Gata4 / light histogram). Normalized against Gapdh and compared vs day CagipGFP-Cagip that was arbitrarily set to 1. Statistical significance was analyzed by ANOVA with post-hoc Dunnett's test (*P<0.001, ***P=0.0001, ****P<0.0001, n = 3, mean ± s.e.m., *n* = 3).

The possibility of cardiac cell commitment was determined by the expression of gene markers like TroponinT (*Tnt*) and cardiac myosin heavy chain (αMHC), and by

counting the contractile foci in colonies formed during differentiation process of cells (Figures 4.40 and 4.41). We observed that cardiomyocyte commitment in mESC overexpressing Gata4 have a decrease in TnT and *αMHC* gene expression compared to those overexpressing Cited2. Moreover, cultures overexpressing both proteins, FlagCITED2 and Gata4, present similar transcripts levels as ones observed in cultures that only overexpress Gata4 (Figure 4.40). Consistently, lower quantities of contractile foci were found in cultures overexpressing either Gata4 or both proteins, CITED2 and Gata4 (Figure 4.41). Despite being preliminary results, this gives us an idea of where CITED2 could be acting in promoting cardiogenesis, which is downstream, Gata4.

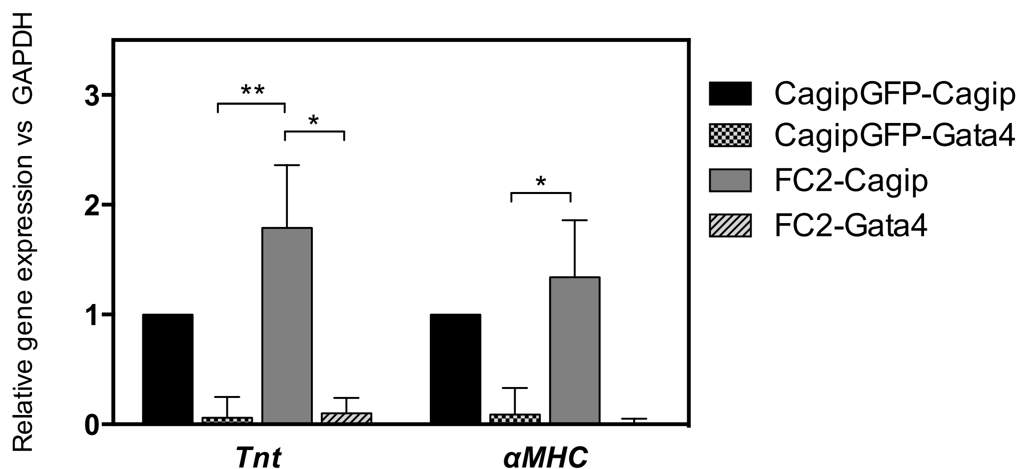


Figure 4.40. Cardiomyocyte gene markers in ESC differentiated cultures at day 12, in gain-of-CITED2-Gata4-function experiments.
Relative gene expression in control cultures (CagipGFP-Cagip/ black histogram); cultures overexpressing Gata4, (CagipGFP-Gata4/ dark dotted hisogram); cultures overexpressing CITED2 (FC2/ gray histogram); and cultures overexpressing both CITED2 and Gata4 (FC2-Gata4 / light histogram). Normalized against Gapdh and compared vs day CagipGFP-Cagip that was arbitrarily set to 1. Statistical significance was analyzed by ANOVA with post-hoc Dunnett's test (*P=0.01, **P<0.0001, n = 3, Mean ± s.e.m., *n* = 3).

Summarizing, the gene expression data together with the contractile foci data, showed that although CITED2 is important for cardiac cell commitment, it was not capable of turn over the endoderm fate by itself in this conditions.

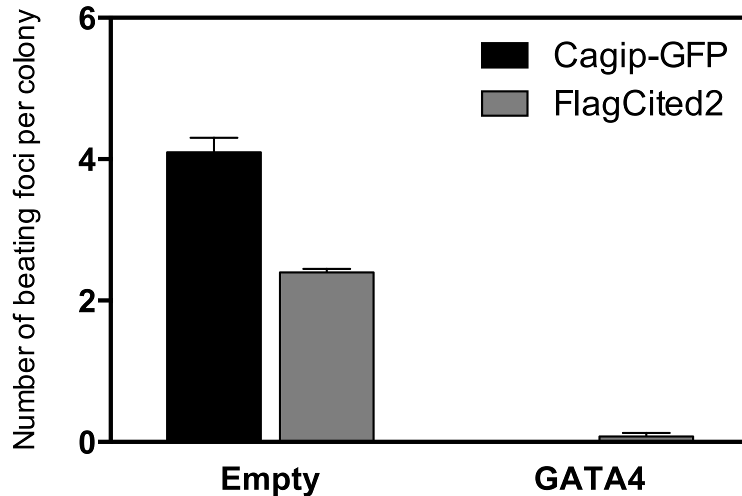


Figure 4.41. Contractile foci in FC2-Gata4 differentiated cultures at day 8.
Cultures transfected with GATA4 vector present a lower number of beating foci per colonies, although some colonies do not present any contractile phenotype when compared with those cultures that have Cited2 constitutively expressed. n = 2

4.6. Functional and physical interacting Cited2 partners

4.6.1. Cited2 is sitting in the Isl1 promoter region

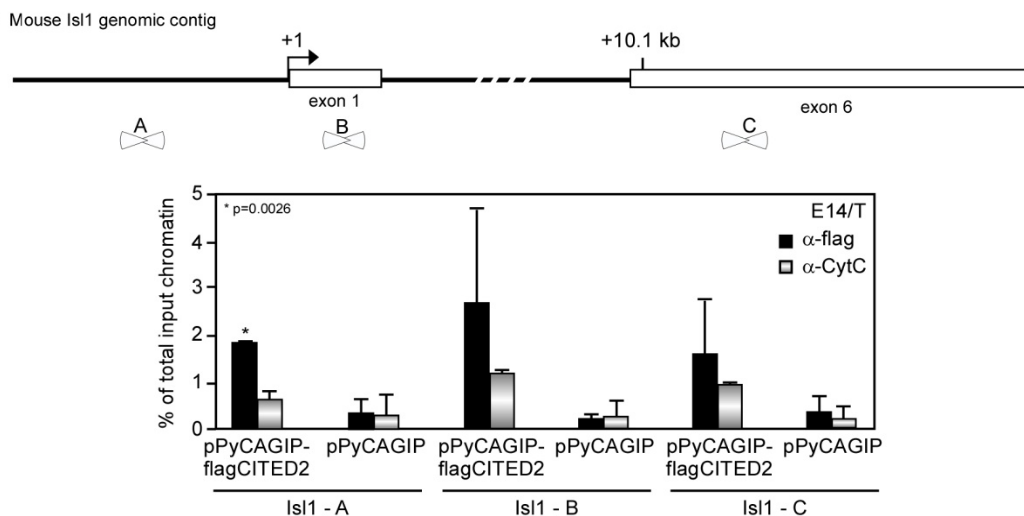


Figure 4.42. Cited2 enrichment in Isl1 genomic sites.
Upper figure, Mouse *Isl1* genomic sites analyzed: A, promoter region; B, exon 1; C upstream region, exon 6. Lower figure, qRT-PCR ratios reflect the enrichment of indicated mouse Isl1 genomic sites (Isl1 –A, -B, -C). mES E14/T cells overexpressing Flag-Cited2 and pPyCAGIP plasmids were subjected to ChIP with anti-flag (black histograms) or anti-cytochrome C (gray histogram) antibodies. The error bars correspond to SEM of triplicates and statistically significant values using a significance t-Student test were calculated, *p=0.0026.

Since flag-CITED2 overexpressionpromoted the expression of *Isl1*, *Nkx2.5* and *Gata4* in E14/T cells (section 4.3.4), we hypothesized that Cited2 might contribute to

the transcriptional regulation of these genes and might even act directly on the regulatory elements of these genes.

In order to determine whether the promoters of *Isl1*, *Nkx2.5*, *Gata4* and *Tbx5* were interacting with Cited2, we performed a chromatin immunoprecipitation (ChIP) assays in E14/T undifferentiated cells expressing flag-CITED2 and compare the results with control cells (figure 4.42).

We observed a significantly enrichment of Cited2 in the promoter region of *Isl1* (primer set Isl1-A) in cells overexpressing flag-Cited2 when compared either to control cells or immunoprecipitation control (figure 4.42). These results suggested that a forced expression of Cited2 in mES cells would promote the recruitment of Cited2 at the promoter of the *Isl1* gene, but not to the proximal promoters of *Nkx2.5*, *Gata4*, *Tbx5* (figure 4.43). However, we cannot rigorously exclude that Cited2 does not interact with other regulatory elements of *Nkx2.5*, *Gata4*, *Tbx5* genes that were not tested in our assays.

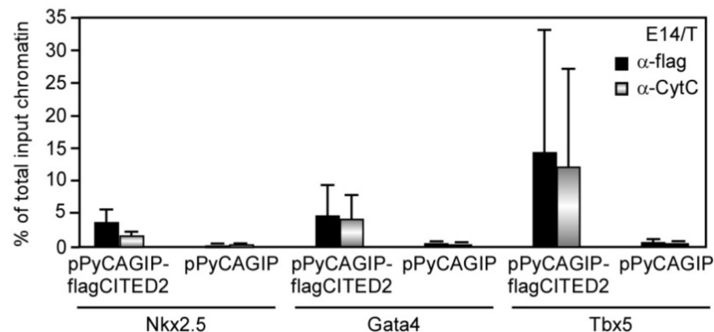


Figure 4.43. Cited2 enrichment at cardiac progenitor promoter regions.
qRT-PCR ratios reflect the enrichment of Cited2 at indicated mouse promoter regions of *Nkx2.5*, *Gata4* and *Tbx5* genes. mES E14/T cells overexpressing Flag-Cited2 and pPyCAGIP plasmids were subjected to ChIP with anti-flag (black histograms) or anti-cytochrome C (gray histogram) antibodies. The error bars correspond to SEM of triplicates and statistically significant values using a significance t-Student test were calculated.

4.6.2. GST-Pull down assay of ISLET1 LIM-domain and CITED2

Preliminary results obtained in the research group indicated that Cited2 interacts *in vitro* with several LIM-containing proteins including Isl1 (Figure 4.44). Furthermore, Cited2 interacts with the Lin11/Isl1/Mec3 (LIM)-domain of Lhx2[98].

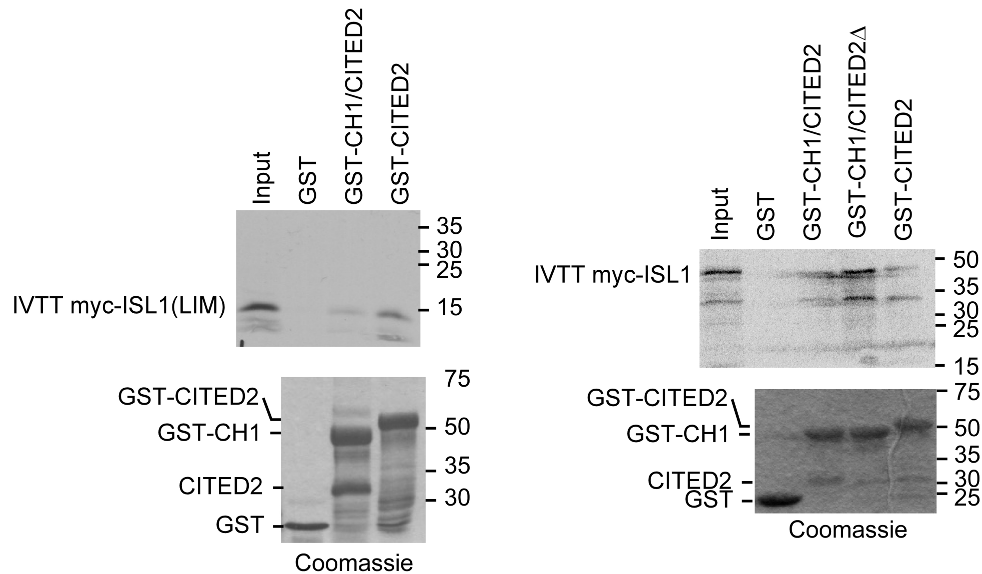


Figure 4.44. In vitro interaction between Cited2 and Isl1.
(Left upper panel) S-labeled IVTT myc-ISL1(LIM) binds GST-CH1/CITED2 and GST-CITED2 immobilized on glutathione beads. (Right upper panel) In vitro-labeled IVTT myc-ISL1 protein binds CITED2 immobilized on beads. The input 10% lane was loaded with 1/10 of the volume of radioactively labeled fusion protein used during the assay. (Lower panels) Coomassie staining. Numbers represents the protein molecular weight.

The figure 4.44 shows the result of one representative experiment where the proteins GST-CITED2 and GST-CH1 produced simultaneously with CITED2 or CITED2 (lacking the CH1-interaction domain) were expressed in bacteria, immobilized on glutathione-agarose beads, and tested for their interaction with the Isl1-LIM domain or the entire Isl1 protein produced in vitro using rabbit reticulocytes. Isl1-LIM domain was specifically retained on beads covered with the GST-Cited2 fusion product compared to background binding to GST alone (Figure 4.44, left panel). Similar results were obtained with entire Isl1 protein (Figure 4.44, right panel).

4.6.3. Co-Immunoprecipitation of Cited2 and Isl1

Cited2 interacts with the Lim11/Isl1/Mec3 (LIM)-domain of Lhx2 [93]. Moreover, preliminary data obtained by our research group indicates that Cited2 interacts *in vitro* with several LIM-containing proteins including Isl1 (GST-Pull down assays, previous *section 4.6.2*). In order to further confirm the CITED2-ISL1 physical interaction, we have set up immunoprecipitation assays (IP). After a phase of antibody optimization, we decided to use anti-flag tag agarose beads (ANTI-FLAG M2 affinity gel, Sigma) to perform the precipitation. HEK-293T cells were co-transfected either with Flag-CITED2

or Myc-ISL1 expressing plasmids, 48h before the protein extraction. The immunoprecipitation was performed overnight at 4ºC, the resulting precipitated was revealed by imunnoblotting (IB) against anti-flag tag and anti-myc tag antibodies (Figure 4.45).

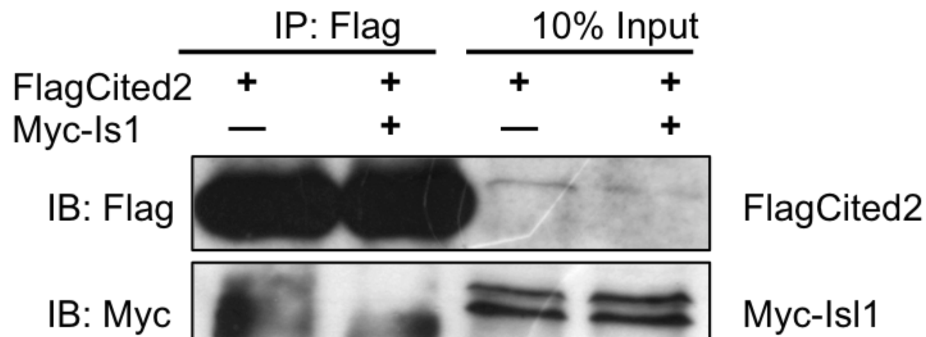


Figure 4.45. Cited2 and Isl1 physical interaction in HEK-293T.
Immunoblotting (IB) of Myc-ISL1 by using an anti-flag covered agarose beads for immunoprecipitation (IP). Protein lysate from cells transfected with pPyCAGIP-FlagCited2 or pCDN3-MycIsl1 are presented. The input 10% lanes were loaded with 1/10 of the volume of protein used during the assay. IP: anti-flag, 1:500. IB: anti-Cited2, 1:5000, 29 *k*Da; anti-myc, 1:5000, 39 *k*Da.

Although the experiments were carried out several times, we still have some troubleshooting during the immunoprecipitation. Occasionally, the transfection seems to occur just fine, however detecting both overexpressing proteins by IB is not as easy as expected. Here in figure 4.45, although the myc-ISL1 was detected in both conditions, transfected and untrasnfected; and Flag-CITED2 protein was not detected, no even in cells transfected just with CITED2 overexpressing vector nor in cells co-transfected.

So at this moment, by using immunoprecipitation technique we cannot conclude that both proteins are interacting physically in the cells, further work is needed. For addressing it I will probably use a different vector for ISL1 overexpressing protein like a construct in pPyCAGIP vector that has a strong driving promoter (might be beneficial). Another approach will be by immunoprecipitate the BiFC complex formation with an anti-YPF antibody (next section 4.6.4).

4.6.4. Bi-molecular fluorescence complementation

We have demonstrated that Cited2 is required for the optimal differentiation of cardiac cell lineages from mESC (Section 4.2 and 4.3), and is co-expressed with Isl1 cells during this process (Section 4.4). Here, we have developed a Bi-Fluorescence complementation assay (BiFC) to further confirm the interaction between Cited2 and Isl1 *in vivo*.

The BiFC assay based on the assembling of two independent complementary domains of a fluorescent protein that are fused to interacting peptides, allowing the visualization of protein interactions in living cells with accurate spatial resolution[161]. BiFC-based strategies were recently established to investigate protein interaction in live human cells[162].

We have modified pPyCAGIP vector (puromycin resistant gene)[101, 102, 114] to express a chimeric protein consisting of the *C*-terminal fragment (residues 156–239) of Venus-YFP (VEC-Yellow Fluorescence Protein) fused to a flag-tagged CITED2 at its *N*-terminal region (VEC-CITED2). We have also prepared a modified pPyCAGIP vector expressing the *N*-terminal fragment (residues 1–155) of Venus-YFP (VEN) fused to the CH1 domain of p300, which is the domain of p300 interacting with CITED2 with high affinity, and used as a control plasmid (VEN-CH1). Finally, we have constructed a pPyCAGIP-VEN-Isl1 vector expressing the *N*-terminal fragment (residues 1–155) of Venus-YFP (VEN) fused to fused to a myc-tagged ISLET1 (VEN-ISL1). These vectors were tested, sequenced and transfected in E14/T cells. Fluorescence in transfected cells will be originated by the close proximity of the residues 1–155 and residues 156–239 of Venus-YFP. Cells harbouring the plasmids were selected for their resistance to puromycin and for the emission of fluorescence.

As expected, a strong and specific interaction between VEN-CH1 and VEC-CITED2 in E14/T cells was detected, panel D in figure 4.46. This interaction was tested to confirm the efficiency of the BifC assay in our cells.

The co-transfection with VEC-CITED2 and VEN-ISL1 vectors, panel C in figure 4.47, also resulted in the specific detection of fluorescent cells. However the majority of mESC do not show YFP^{+} fluorescence, we believe that this might be due to the efficiency of transfection, or to the fact that cells were only transfected by one plasmid but still are efficiently resistant to puromycin.

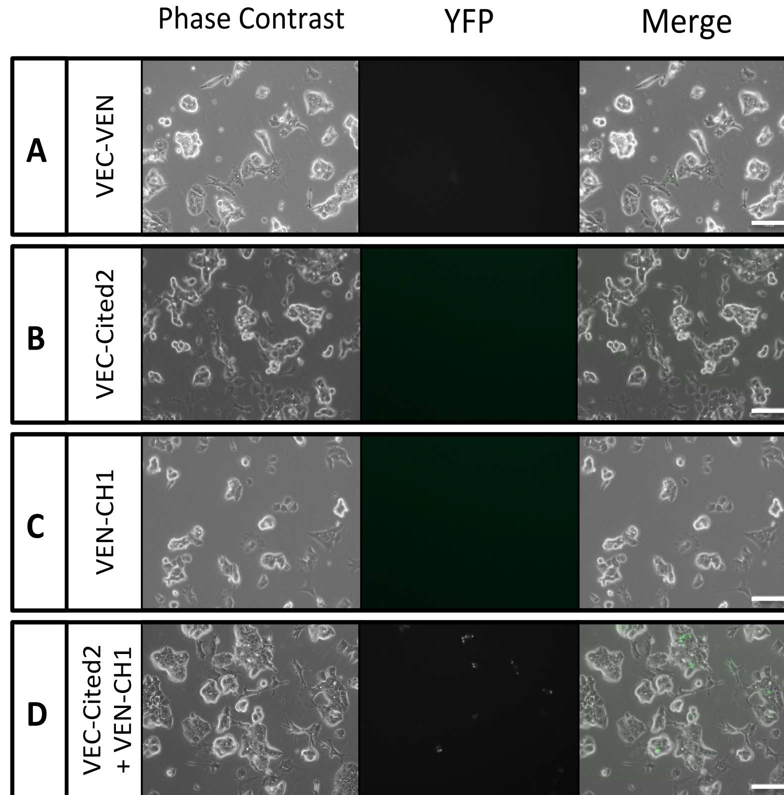


Figure 4.46. Bimolecular fluorescence complementation between VEC-CITED2 and VEN-CH1 in E14/T cells. Representative microphotographs, phase contrast (left), YFP (middle) and merged images (right) of E14/T cultures transfected with 250 *n*g of individual vectors (A) empty VEC and VEN vectors, (B) VEC-CITED2, (C) VEN-CH1; (D) VEC-CITED2 and VEN-CH1 vectors. Bars, 100 μm.

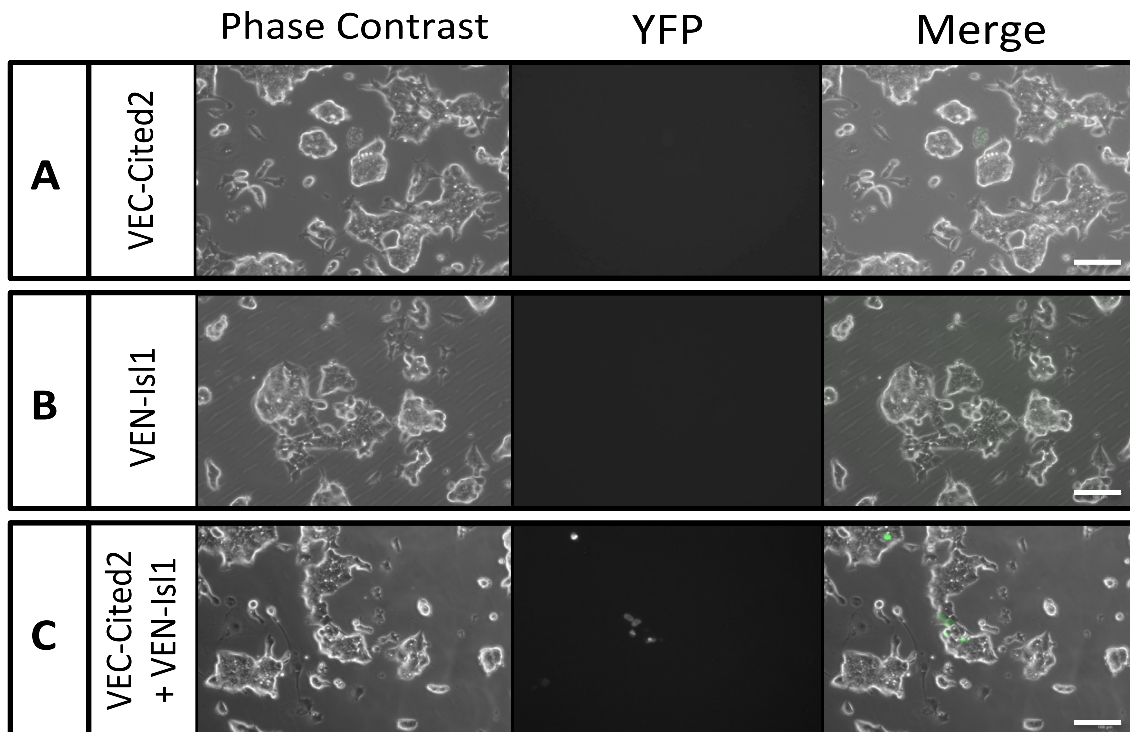


Figure 4. 47. Bi-molecular fluorescence complementation between VEC-CITED2 and VEN-ISL1. Representative microphotographs, phase contrast (left), YFP (middle) and merged images (right), of E14/T cultures transfected with 250ng of individuals vectors (A, B) or co-transfected (C): (A) VEC-CITED2, (B) VEN-SL1, (C) VEC-CITED2+VEN-ISL1. Scale bar, 100 μm.

Nevertheless, the BiFC approach indicated a CITED2-ISL1 interaction in pluripotent mESC. However, if Cited2 and Isl1 are cooperating to differentiate ESC towards cardiac progenitors and to sustain their self-renewal, we expect this interaction to be maintained, and even increased during cardiac progenitor stage. To test this hypothesis, we performed a preliminary experiment in which mESC were transfected with both vectors and were subsequently differentiated (Figure 4.48).

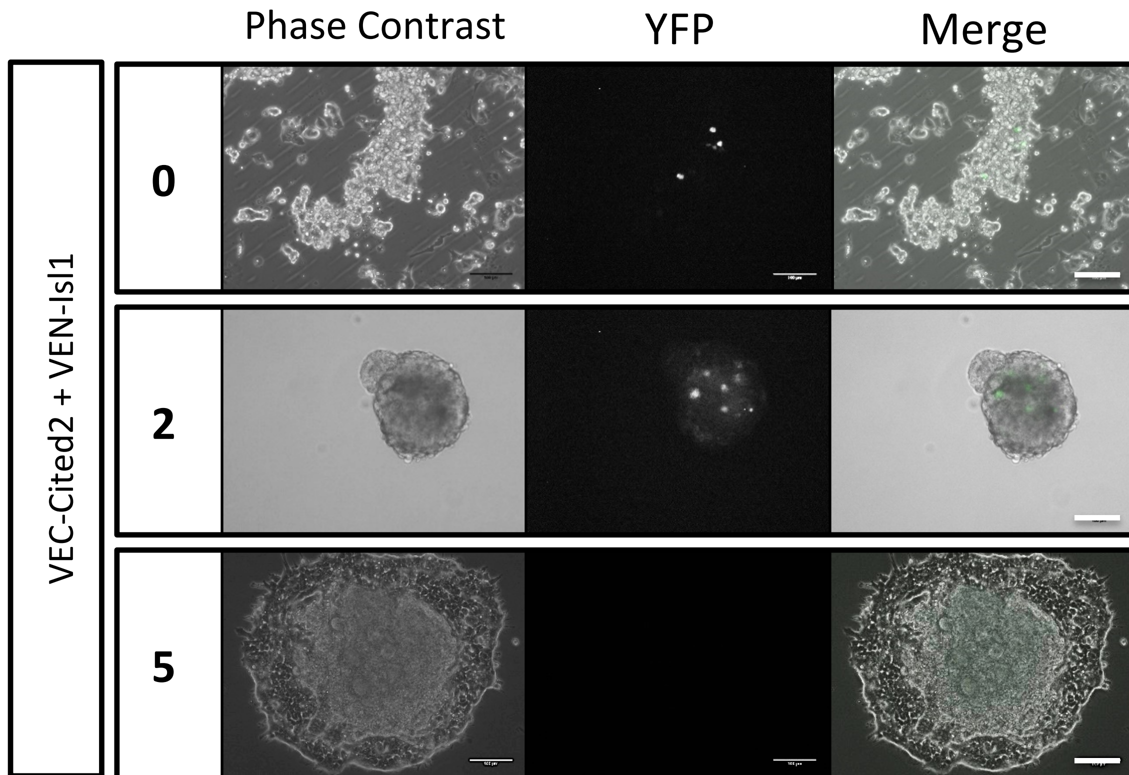


Figure 4.48. BiFC during differentiation cultures between VEC-CITED2 and VEN-ISL1.
Representative microphotographs, phase contrast (left), YFP (middle) and merged images (right), of E14/T cultures transfected with 250ng of individuals vectors (A, B) or co-transfected (C): (A) VEC-CITED2, (B) VEN-SL1, (C) VEC-CITED2+VEN-ISL1. Scale bar, 100 μm.

We found that the fluorescent signal resulting from the potential CITED2-ISL1 in differentiating ES cells was stronger at day 2 of differentiation and almost disappeared by day 5 (Figure 4.48). However, the fluorescent signal was not present in all EBs formed. We allowed cultures to differentiate for 12 days of differentiation, and we found more contractile foci in cultures that were transfected with individual plasmids expressing either VEC-CITED2 or VEN-ISL1, in comparison to cultures transfected with control vectors or both (Figure 4.49A). The result suggest that no synergistic effect on the emergence of beating foci when VEC-CITED2 and VEN-ISL1 were co-transfected,

besides the observation that *αMHC* gene expression was enhanced in those cultures (Figure 4.49).

Furthermore, only VEC-CITED2 or VEN-ISL1 or both transfected cultures, presented similar gene expression behaviour of during differentiation (Figure 4.50). However, the high expression of β-III-Tubulin in cultures transfected with both vectors suggest ectodermal commitment (Figure 4.50E), although at this moment is only a hypothesis that remains to be elucidated.

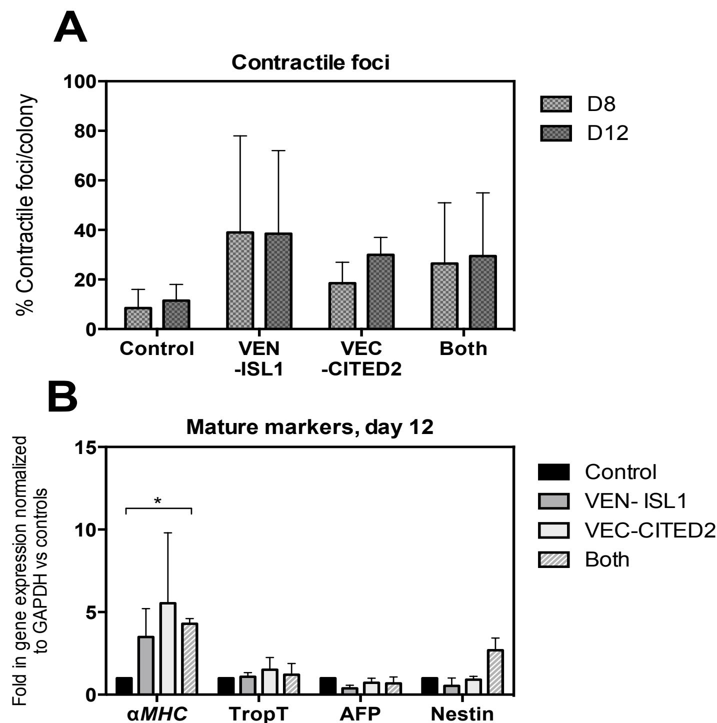


Figure 4.49. VEC-CITED2 and VEN-ISL1 differentiated cultures.
(A) Contractile foci in differentiated cultures transfected with: control, VEN-ISL1, VEC-CITED2, or both vectors, were counted at differentiation day 8 (light histogram) or day 12 (dark histogram). The error bars correspond to SEM of n=2. (B) Mature gene markers: *αMHC, TropT, AFP* and *Nestin,* analyzed at day 12 of differentiation in cultures transfected with: control (dark histogram), VEN-ISL1 (dotted histogram), VEC-CITED2 (light histogram) or both (stripped histogram) vectors. Fold in gene expression were calculated setting arbitrarily to 1 for each gene. The error bars correspond to SEM of n=2. The (*) asterisk correspond to statistically significant values using a significance t-Student test, $p<0.05$.

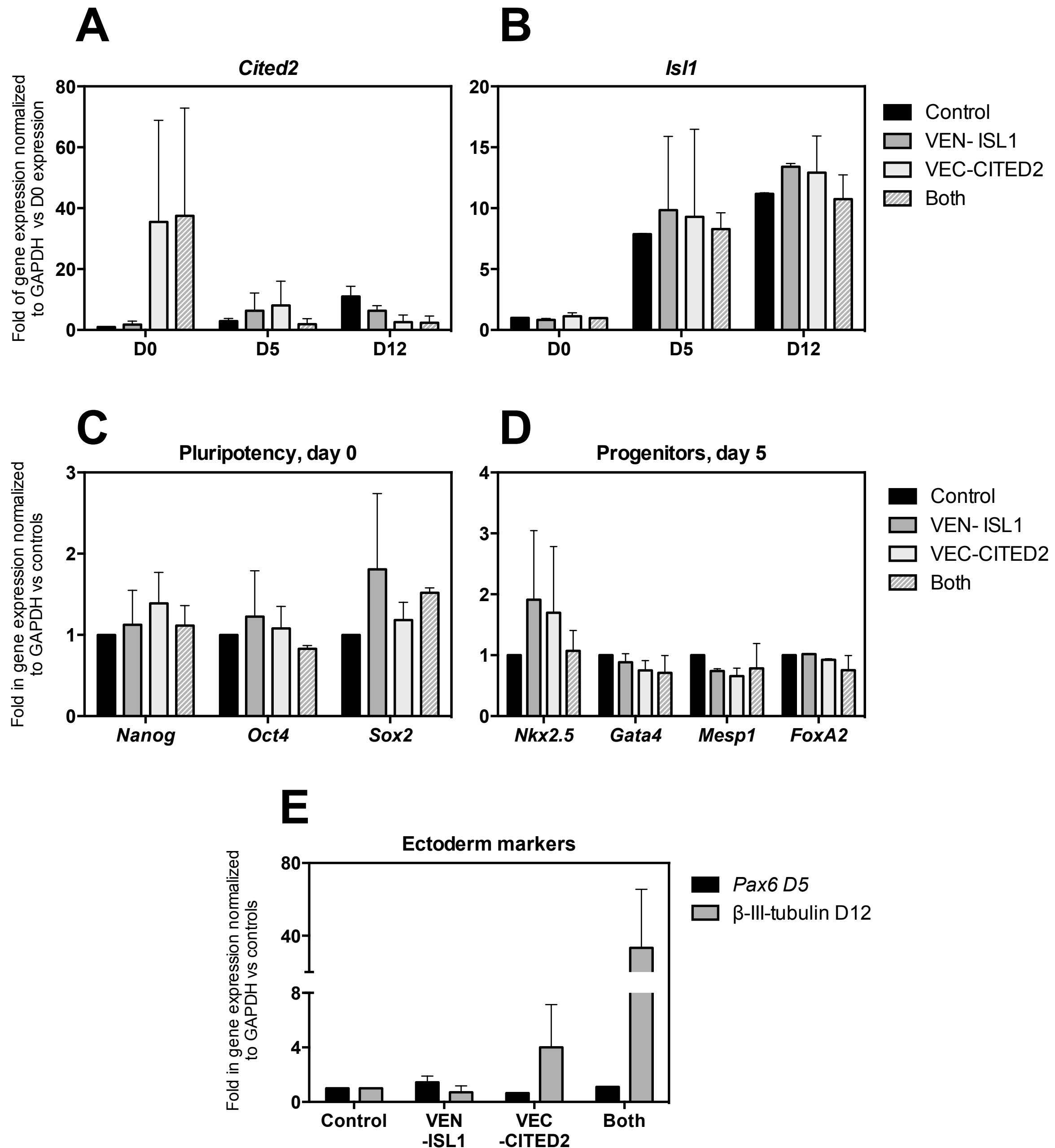


Figure 4.50. VEC-CITED2 and VEN-ISL1 gene expression profile during differentiation.
Differentiation in cultures transfected with: control (dark histogram), VEN-ISL1 (dotted histogram), VEC-CITED2 (light histogram) or both (stripped histogram) vectors. Differentiation days (D) are indicated. Fold in gene expression were calculated setting arbitrarily to 1 the day 0 expression (A, B) or control gene expression for each gene (C,D,E). Relative gene expression of: *Cited2* (A); *Isl1* (B); *Nanog*, *Oct4* and *Sox2* (C); *Nkx2.5*, *Gata4*, *Mesp1*, *FoxA2* (D). (E) Ectoderm gene markers, *Pax6,* analyzed at day 5 (dark histogram) and *b-III-Tubulin,* analyzed at day 12 (gray histogram). The error bars correspond to SEM of *n*=2.

Since the number of mESC with YFP^+ signal was low for ESC co-transfected with CITED2-ISL1, we decided to isolate and to enrich this population after transfection by taking advance of its ES cell clonogenic property. The isolation of YFP^+ cells from CITED2-ISL1 cultures was performed manually until the 5th cell passage the cells were

sorted and maintained under pluripotency conditions (Figure 4.50). The resulting isolated cells were not able to maintain its pluripotency state, the day after being sorted they started to differentiate by presenting an alteration in cell morphology. However, nor transcripts neither protein profile were examined in the resulting cells, we cannot conclude that this behaviour was due to the CITED2-ISL1 interaction.

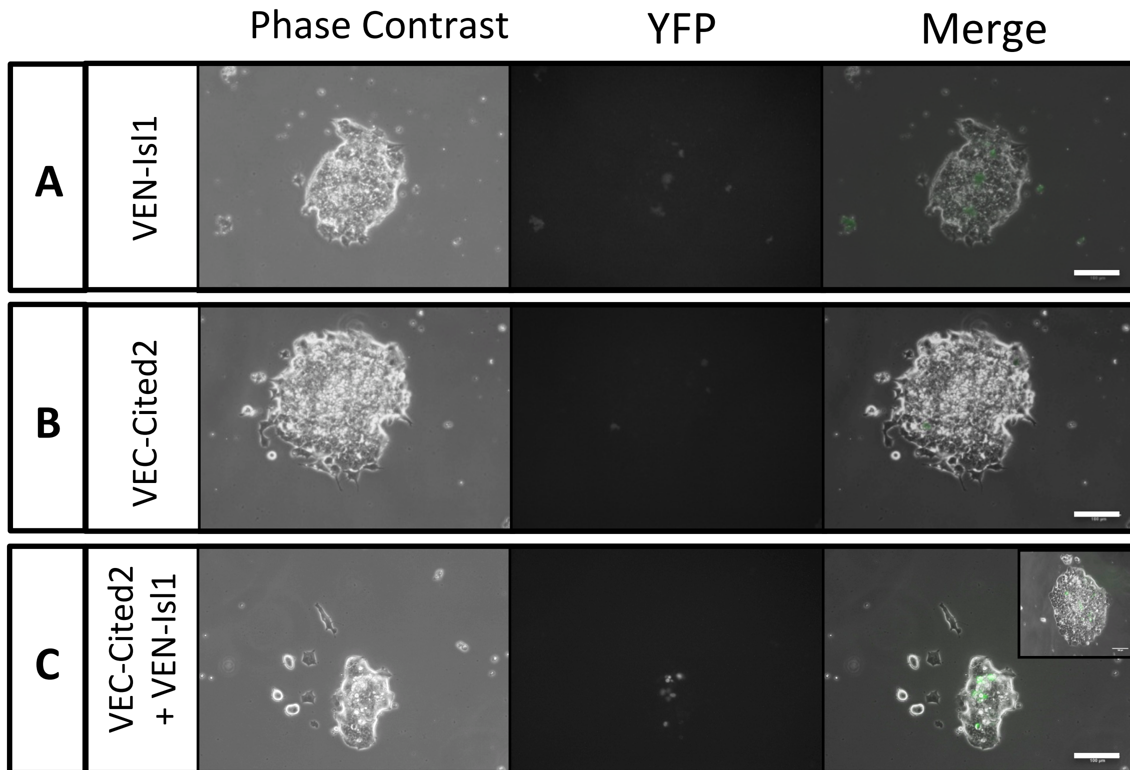


Figure 4.50. Isolated mES colonies by YFP fluorescence derived from VEC-CITED2 and VEN-ISL1 interaction. Representative microphotographs, phase contrast (left), YFP (middle) and merged images (right), of isolated colonies derived from E14/T cultures, maintained during 7 days under pluripotency conditions. Cells were transfected with individual vectors (A, B) or co-transfected (C): (A) VEC-CITED2, (B) VEN-SL1, (C) VEC-CITED2+VEN-ISL1. Scale bar, 100 μm.

CHAPTER V

DISCUSSION

5. Discussion

5.1. *Cited2 promotes the specification of the primitive streak to mesoderm and endoderm in mouse embryonic stem cells.*

Embryonic stem cell differentiation comprises a powerful system to explore the molecular mechanism underlying cardiac cell fate decisions and a potential source of cells for future cardiovascular therapy in humans[36].

In this thesis we demonstrated that Cited2 is required for an efficient differentiation of ESCs towards cardiac cell lineages. Moreover, Cited2 might take part in several steps that mark the commitment of pluriptent ESCs to mature cardiac myocytes, starting with the early specification of ESCs towards cardiac cell mesoderm cell fate. Indeed, the reduction of Cited2 expression by knockout approaches led to the downregulation of the expression of *Brachyury* that acts during early primitive streak specification[58], and is an early marker of ESC differentiation towards the mesoderm germ layer. A similar effect of Cited2-knockout in ES cells was previously reported[106]. The transition from ESCs with pluripotency to mesodermal characteristics is critical for the differentiation of ES cells towards cardiac lineages. Therefore, we propose that the *Brachyury* dysfunction upon Cited2 knockout in differentiated cultures, marks a reduction of differentiation of ESCs into mesoderm cells, and consequently, cells depleted for Cited2 will be less prone to differentiate into cardiac cell lineages. Thus, the downregulation of cardiac progenitor gene markers and the reduced number of beating foci observed in Cited2-depleted differentiation culture might be a consequence of the early mesodermal cell differentiation impairment.

Our results suggest that the expression of *Brachyury* is reduced in Cited2-depleted differentiating ESC, although, we have no clear idea about the molecular mechanism behind this regulation, these observations suggested that Cited2 prevails and is involved in (directly or indirectly) *Brachyury* expression. Interestingly, MacDonald and colleagues 2008, have performed a conditional knockout of Cited2 in all cells expressing Brachyury during mouse embryonic development and observed only

infrequent and minor heart developmental defects in opposition to the epiblastic knockout of Cited2 which resulted in consistent and pleiotropic heart defects in every Cited2 knockout embryo[112]. These results suggested a minor role for Cited2 in mesodermal derivatives for heart development. However, in light with the results that we have now obtained in Cited2-knockout ESC, we propose that Cited2 might be necessary to establish the expression of *Brachyury* and for mesodermal differentiation/development from pluripotent tissues and once the mesodermal tissue is differentiated, the role of Cited2 is less critical.

The role of Cited2 in the expression of *Brachyury* in ESC differentiation has not been investigated to date. However, studies of mouse embryonic development have showed that a trophoblast derivative, the extraembryonic ectoderm, provides signals to promote *Brachyury* expression in the epiblast. The induction of thophoblastic cells differentiation from hES cells depends on the inhibition of TGF-β/Activin/Nodal signaling[163]. Later, the mesoderm formation and patterning rely on Nodal/Smad2-3 signaling in mouse the development[164], hES[165] and mES cells[166] differentiation. Interestingly, Cited2 was showed to be important for the development of the epiblast derivative tissues[112] and for controlling the left-right patterning, as well as heart development via Nodal-Pitx2c pathway[94]. Nodal overexpression in mESC was shown to promote their differentiation into mesoderm and endoderm cell derivatives by up regulating specific gene markers (i.e. AFP, Brachyury, Flk1)[167]. Our observations also indicated that Cited2 gain-of-function enhances Nodal expression and correlated with an increased expression of αMHC and Tnt, as well as Flk1.

Moreover, our results are consistent with a recent study of Cited2 suggesting the dual role of Cited2 during pluripotency and differentiation of ES cells. Li and colleagues (2012) have reported that established Cited2-knockout cell lines were also impaired in their ability to differentiate into cardiac cell lineages amongst other cell fates. They proposed that depletion of Cited2 could sustain mESCs pluripotency as well as affect the differentiation of mESCs by a delay in the silencing Oct4 and Nanog expression upon the start of differentiation[106]. They even showed a direct repression effect on Oct4 gene expression by Cited2, which would account for the sustained expression of Oct4 in Cited2-knockout ESCs submitted to differentiation, delayed the differentiation process.

To demonstrate the role of Cited2 in ESC differentiation, we have employed a distinct strategy, and established novel ESC lines allowing the conditional deletion Cited2 alleles in ESC by addition of 4-hydroxy tamoxifen to the cell culture. In essence, what we are observing in our experiments is the immediate effect of Cited2 depletion in ESC functions, whereas Li and colleagues have established cells lines that might have adapted to function without Cited2 alleles and might have acquired compensatory mechanisms to overcome the absence of *Cited2* expression. In any case, in our experimental set up we did not observe any impairment of Oct4 and Nanog silencing in the initial days of differentiation, concluding that Cited2 is important for maintenance of pluripotency and the proper down-regulation of *Cited2* expression is crucial for differentiation to proceed. We have shown that dosage of Cited2 expression in SHF-progenitors might also be determinant for their maintenance of the renewal state. Our results in SHF-progenitors demonstrated that a higher *Cited2* expression levels the lower *Isl1* expression. Have been suggested that *Isl1* downregulation induced by β-catenin was necessary for Wnt-β-catenin-induced expansion of SHF-progenitors, suggesting that higher Isl1 levels could promote differentiation triggers further development of SHF-progenitors into cardiac cells rather than promoting its renewal state[168], suggesting that Cited2 might mark a more multipotent cardiac progenitor.

We have demonstrated that Cited2 is also critical to maintain stemness of ESCs (Kranc et al. – manuscript submitted to STEM CELL REPORTS). The impairment of the differentiation ability that we observed in E14/T cells constitutively overexpressing high levels of flag-CITED2 in the presence of puromycin, also corroborates role of Cited2 in the maintenance of pluripotency. Interestingly, the time course of Cited2 transcript expression during ESC differentiation revealed that Cited2 expression is decreased in the first two days after differentiation compared to its levels in pluripotent ESC, and is increased again from the third day of differentiation onwards. This observation suggests a dynamic and biphasic expression of Cited2 during ESC differentiation, and the levels of Cited2 expression have to decrease to allow ESCs to reach the initial step of differentiation, probably leading to mesoderm differentiation and rise again for the optimal cardiac differentiation process of ESC.

Our results validated previous findings by showing that the constitutively Cited2 expression in ES cells enhanced *Nodal* transcription, as well as the expression of *Isl1*, *Gata4*, *Nkx2.5* which are markers of multipotent cardiac progenitors emerging from the left-lateral plate mesoderm, and support the notion that Cited2 is necessary for the initiation and/or continuation of Nodal expression in the lateral plate mesoderm[111]. Since Cited2 is expressed in the lateral-plate mesoderm[77, 111, 112], and its absence leads to loss of *Nodal*-activated transcripts such as *Nodal itself*, *Lefty2* and *Pitx2c* in the left-lateral plate mesoderm[112].

The overexpression of Cited2 in ESCs from day 0, also promoted an increase in the expression of *Nodal* at day 6 of differentiation.

5.2. Cited2 promote multipotent cardiovascular progenitor specification from mouse embryonic stem cells.

We revealed that Cited2 stimulates the specification of multipotent cardiovascular progenitors from the primary and secondary heart fields. This was supported by the up regulation of markers common in both (*Nkx2.5*, *Gata4*) and specific for the primary (*Tbx5*) and the secondary heart field (*Isl1*).

The transcriptional machinery during multipotent cardiovascular progenitor stage is tightly regulated by Nkx2.5, Isl1, Gata4 and Tbx5. Gata4 contribute to the activity of *Isl1* enhancer in the developing heart[169]. *Mef2c* expression is induced by most of transcription factors marking all cardiac progenitor, such as Isl1[158]. *Mef2c* promoter contains Gata4 and Isl1 activation sites[158 170]. Moreover, Mef2c induces the expression of cardiac muscle genes for structural proteins and myosin heavy chain at the differentiation stage[171].

T-box transcription factor, Tbx5 marks the transition from primordial muscle type to chamber myocardium[131]. During this transition, Tbx5 acts as an activator of atrial natriuretic factor (*Nppa*) gene[132]. Also, *Tbx5* have been shown to be required for cardiac function and for full differentiation into contracting cardiomyocytes in transdifferentiated cultures[44]. Our results showed that Cited2 is required for normal *Tbx5* and its target *Nppa* gene expressions. Furthermore, Tbx5 regulates *aMHC* and *Tnt*

during cardiac division, by controlling cardiac cell cycle associated G_1/S-phase proteins in early heart development[133]. Our result suggests that Cited2 also has an effect over cardiac cell cycle by regulating *Tbx5* and *cyclin-D2* expression.

Furthermore, our results pointed out that Cited2 knockout generally affected T-box protein expression at different cardiac differentiation stages. Indeed, *Brachyury* was affected by Cited2 depletion at initial stages of differentiation and *Tbx5,* which is involved in the chamber patterning in mature heart at later stages, day 5 and day 12 essentially. The T-box transcription factor *Eomes* acts cooperatively with Nodal/Smad2/3 in the epiblast, where the low levels of Nodal are necessary to activate *Eomes* and induce cardiac mesoderm formation[136]. Moreover, results suggested that Cited2 could be a target of Eomes, Smarca4 and Tcfap2c during cell renewal of the trophoblast stem (TS) cells[172]; although Cited2 is not expressed in TS cells, its expression has been associated with the onset of differentiation towards trophoblast giant cells, spongiotrophoblast and glycogen cells during placental development[107]. *Eomes* expression is sufficient to activate *Mesp1* and promote the migration of a discrete mesodermal population in the cardiac fields[136], our results point that probably Cited2 is being part of the Eomes-Nodal signaling. Moreover, we have shown that Cited2 regulates directly the expression of Tbx3 in mouse pluripotent ESCs (Kranc et al. – manuscript submitted to STEM CELL REPORTS). Moreover, during heart development in mouse and humans, Tbx3 is required for the development of the cardiac conduction system[173].

Our study demonstrated that Cited2 promotes and maintains the expression of second heart field cell-type population marked by Isl1. We also observed that the overexpression of Cited2 in pluripotent ESCs increased the expression of transcripts encoding *Gata4*, *Nkx2.5* ad *Isl1*. We have shown by ChIP experiments that Cited2 binds to the promoter region of Isl1 in these undifferentiated ES cells, suggesting that Isl1 gene is a direct target of Cited2. However, it would be of interest to determine whether Cited2 also interacts with the promoter of Isl1 when expressed at endogenous levels, and also in more relevant cell subtypes for cardiogenesis, such as the cardiac progenitors themselves. We have also attempted to determine the effect of Cited2 on the activity of the Isl1 promoter by transient transfection assays in mouse ESC, in

HEK293 cells and the in hepatoma Hep3B cells lines, but in the conditions that we used Cited2 failed to activate Isl1 promoter, but rather led to a decrease of its transcriptional activity (data not shown). Thus, at the moment it is not clear how Cited2 promotes the expression of the Isl1 transcript in mouse ESC, and why it is recruited to the promoter of Isl1.

On another level, our results also suggest that Cited2 interacts with the LIM domain of Isl1, as we observed by GST pull-down and BifC assays. The overexpression of both Cited2 and Isl1 individually in ES cells promoted cardiac differentiation, but the simultaneous overexpression of these factors in ESCs did not show any synergistic effect on cardiac differentiation. Interestingly, the simultaneous overexpression of Cited2 and Isl1 in ESCs, led to a drastic increase in the β-III-tubulin expression, suggesting a differentiation towards neural cells. Apart from its relevance for cardiac development and differentiation, Islet1 is important for neural development, thus the interaction between Cited2 and Isl1 might be relevant for neural ESC differentiation. The dosage of Cited2 and Isl1 protein expression might also be determinant for their cooperative function. Indeed, our preliminary results have indicated that co-transfection of plasmids expressing Isl1 and Cited2 at low concentration in Hep3B cells would act to activate the promoter of Mef2c, whereas elevated concentrations of Cited2-expressing plasmid with the same Isl1 plasmid would lead to an inhibition of the Mef2c promoter activity (data not shown). In the experiments presented in this work, our approaches did not allow to control the amount of Cited2 and Isl1 overexpressed, leading perhaps in the failure to demonstrate a clear functional cooperation between Isl1 and Cited2 to promote cardiac cell fate. Recently, the protein Ajuba, a LIM domain protein has been characterized and identified as a crucial regulator the SHF progenitors specification and expansion, probably by binding to Isl1 and negatively modulating its transcriptional activity[174]. Since Ajuba is a LIM-domain protein interacting with Isl1, it would be of interest to determine the effect of Cited2 on the interaction between Isl1 and Ajuba, and the consequences on SHF progenitors specification, expansion and differentiation.

Previous results showed that Cited2 binds to the LIM domain of other transcription factor, such as Lhx2[93] and Lmo4, a LIM-only (LMO) protein[175]. Proteins containing LIM domains function as transcriptional regulators, and this domain may act

as a molecular adaptor for multiprotein complexes. Moreover, the "four-an-a-half LIM domain" (FHL 1-4) proteins have an amino-terminal half LIM domain followed by four full LIM domains in tandem. Interestingly, three members of this family (FHL 1-3) regulate the transcriptional activity of the hypoxia-inducible factor (HIF-1), which coordinates the adaptive responses to hypoxia, although only one member of this family FLH2 binds physically to HIF-1α. However, the FHL1 protein disrupts the binding of HIF-1α to p300/CBP, acting in a similar manner as Cited2[79], supporting the hypothesis where Cited2 could interact with LIM domain proteins and regulate gene expression either by directly interaction or by disruption of p300/CBP interactions.

P300 has been showed to facilitate the Nkx2.5 transcriptonal activity[176], and P300 co-occupies specific cardiac enhancers together with Gata4, Nkx2.5, Tbx5 and Mef2c and might collaborate with those factors to direct cardiac gene expression[170]. Despite, the lack of detection of the presence Cited2 on Gata4, Nkx2.5 and Tbx5 promoters in our experiments by ChIP, we cannot exclude the possibility that Cited2 might be recruited to some other region other than those tested within these promoters. Since Cited2 is tightly binding to p300 and CBP, it would be of interest to test the presence of Cited2 in the specific cardiac enhancer sites that are co-occupied by p300.

And more importantly, the work we presented contributes to increase the previous and squat knowledge of Cited2 function during cardiac differentiation from mESCs. Li and al, 2012 reported that Cited knockout in ESCs resulted in cardiac cell differentiation impairment, and determined that *Brachyury* expression was reduced. These authors also reported alterations in the normal ectoderm and endoderm derivatives. It was proposed that Cited2 induces cardiomyocyte differentiation by affecting the expression of NFAT3 leading to the down-regulation of Nkx2.5 and βMHC genes[106]. Based on the results exposed here, we propose that Cited2 is important for the mesoderm specification since, as reported by Li and colleagues, we also observed a diminution of *Brachyury* (and *Gsc*) at early stages of ESC differentiation. However, the data presented here using Cited2 gain of function and conditional knockout experiments, indicated also that Cited2 also has additional roles in the cardiac progenitors specification (as suggested by the role in Isl1 expression and its physical

interaction with Isl1) and during proper cardiac maturation (as suggested by the reduction in *Tbx5* expression).

Cited2 might a central mediator of the TGF-beta superfamily, such as Nodal, Activin A and BMPs. Indeed, it has been shown that Cited2 is co-activor of Smad2/3[100] wich are mediating Nodal, activin A signalling pathway. Moreover, Cited2 controls ErbB3 gene expression in neural crest cells[76] and ErBB3 is itself a downstream target of BMP and promotes gliogenesis[177].

Clearly, further studies are needed to more precisely determine the Cited2-bignding sites in cardiac regulatory regions and to determine *in vivo* the contribution of Cited2 during in early cell commitment decisions.

Overall, our results demonstrated that Cited2 expression in ESCs is important for their correct specification towards mesoderm and cardiac mesoderm, for the expression of pro-cardiogenic genes and markers of cardiac progenitors from the first and secondary heart field. Moreover, the overexpression of Cited2 in ESCs stimulated greatly the commitment of ESCs towards cardiac cell fate, thus use of Cited2 protein might be considered as an asset in the specification of pluripotent cells to cardiac cell lineages, and even in transdifferentiation experiments.

Chapter VI

Conclusion

6. Conclusion

Here we demonstrate that CITED2 is important during early developmental stages from ES cells, leading to a proper heart cell development. Furthermore, we confirm previous data and contribute to the current knowledge; taken together the results suggest that CITED2 might act as a pivotal protein during early cell commitment decisions.

By observation during loss-of-function as well as gain-of-functions experiments, we discover that CITED2 is acting in regulating the gene expression of important mesodermal genes. We hypothesized that CITED2 could be acting by mainly two different ways during mesodermal development, and also acting at late stages of cardiac differentiation by inducting the expression of Tbx5 and regulates the appearance of contractile foci.

Firstly, Cited2 by altering the initial step of cardiac differentiation from ES cells, namely Brachyury expression, could be responsible for the lack of cardiomyocytes in Cited2 KO cultures, altering the normal mesoderm program. CITED2 regulates essential mesodermal progenitors, like Nodal, Brachyury, GSC and Mesp1, leading to a signaling cascade in the epiblast inducing the epithelial-mesenchyme transition. Mesp1 is thought to induce pro-cardiogenic factors such as Nkx2.5, Tbx5, Isl1 and Gata4 that define cells as cardiac progenitors.

Secondly, by accumulation of the CITED2 protein during gain-of-function experiment, we observed enhance in gene expression of specific cardiac progenitors markers (Islet1, GATA4, Nkx2.5), as well as an endoderm marker (FoxA2). This mesodermal activation during ES cells lead to an enrichment of cardiomyocytes in differentiating cultures. Moreover, we discovered that CITED2 is found in a specific Islet1 promoter region of during this process. Although, in Nkx2.5 and Gata4 specific promoter regions, CITED2 was not found, this suggest that CITED2 could be acting over these genes in a paracrine manner. We found that CITED2 is important for specification

of cardiovascular progenitors, namely for the proper expression of Nkx2.5 and Tbx5, hence for Mef2c, suggesting that Mef2c is an indirect target of CITED2. Furthermore, the CITED2 expression was also found in all cardiac progenitors, being more expressed on second than in first heart field progenitors. These second heart field progenitors are known to expresses the transcription factor Islet1. The expression of Islet1 in second heart field is maintained by activation of βCatenin, also in this conditions the Cited2 expression was enhanced. The Cited2 knockdown in second heart field progenitors leads to an enhancement of cardiomyocytes, whereas the gain-of-function experiments suggest that the constant Cited2 expression avoids cardiomyocyte commitment. Those results prompt to a hypothesis that CITED2 might be acting in dose-dependent manner through the regulation of cardiac transcription factors during the transition of cardiac progenitor towards fully differentiated cardiomyocytes and, also CITED2 could being regulated by Wnt/βCatenin pathway during cardiac crescent developmental stage by attenuating Cited2 expression in order to proceed with the cardiovascular program.

Moreover, recently results suggest that CITED2 alone or in combination with ISLET1 activate the transcription of MEF2c, although the activation of ISLET1 promoter regions by CITED2 is not evident, the results suggest that this interaction could be tissue specific. In gain of function experiments where the overexpression of CITED2 and Isl1 where conducted at the beginning of differentiation, cardiomyocyte induction where observed.

The overall conclusion of this work here presented is that the mechanism by which CITED2 is responsible for the cardiac phenotype mainly resides during gastrulation and still needed for the proper cardiac differentiation during progenitor stage.

Bibliography

1. Evans M. Discovering pluripotency: 30 years of mouse embryonic stem cells. *Nat Rev Mol Cell Biol*. 2011;12:680-686
2. Evans M, Kaufman M. Establishment in culture of pluripotential cells from mouse embryos. *Nature*. 1981;292:15415-15416
3. Martin G. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A.* 1981;78:7634-7638
4. Bradley A, Evans M, Kaufman M, Robertson E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature*. 1984;309:255-256
5. Schuringa JJ, van der Schaaf S, Vellenga E, Eggen BJL, Kruijer W. Lif-induced stat3 signaling in murine versus human embryonal carcinoma (ec) cells. *Experimental Cell Research*. 2002;274:119-129
6. Burdon T, Smith A, Savatier P. Signalling, cell cycle and pluripotency in embryonic stem cells. *Trends in Cell Biology*. 2002;12:432-438
7. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science*. 1998;282:1145-1147
8. Thomson MAMKCMSIC-PCCPHMAWJI-EJA. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Genomes & Developmental Control*. 2000;227
9. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126:663-676
10. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature*. 2007;448:313
11. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131:861-872
12. Joseph Itskovitz-Eldor MS, Dorit Karsenti, Amir Eden, Ofra, Yanuka MA, Hermona Soreq, Nissim Benvenisty. Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. *Molecular Medicine*. 2000;6:88–95
13. Doetschman TC EH, Katz M, Schmidt W, Kemler R. The in vitro development of blastocyst-derived embryonic stem cell lines: Formation of visceral yolk sac, blood islands and myocardium. *J Embryol Exp Morphol.* . 1985;87:27-45
14. Holtzinger A, Rosenfeld GE, Evans T. Gata4 directs development of cardiac-inducing endoderm from es cells. *Dev Biol*. 2010;337:63-73
15. Ieda M, Fu J-D, Delgado-Olguin P, Vedantham V, Hayashi Y, Bruneau BG, Srivastava D. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell*. 2010;142:375-386
16. Desbaillets I, Ziegler U, Groscurth P, Gassmann M. Embryoid bodies: An in vitro model of mouse embryogenesis. *Exp Physiol*. 2000;85:645-651
17. Burridge PW, Anderson D, Priddle H, Barbadillo Munoz MD, Chamberlain S, Allegrucci C, Young LE, Denning C. Improved human embryonic stem cell

embryoid body homogeneity and cardiomyocyte differentiation from a novel v-96 plate aggregation system highlights interline variability. *Stem Cells*. 2007;25:929-938

18. Michael A Laflamme KYC, Anna V Naumova, Veronica Muskheli, James A Fugate,, Sarah K Dupras HR, Chunhui Xu, Mohammad Hassanipour, Shailaja Police,, Chris O'Sullivan LC, Yinhong Chen, Elina Minami, Edward A Gill, Shuichi Ueno,, Chun Yuan JGCEM. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nature Biotechnology*. 2007;25:1015
19. Evans T. Embryonic stem cells as a model for cardiac development and disease. *Drug Discovery Today: Disease Models*. 2008;5:147-155
20. Dambrot C, Passier R, Atsma D, Mummery CL. Cardiomyocyte differentiation of pluripotent stem cells and their use as cardiac disease models. *Biochemical Journal*. 2011;434:25-35
21. Gratsch TE1 OSK. Noggin and chordin have distinct activities in promoting lineage commitment of mouse embryonic stem (es) cells. *Dev Biol.* . 2002;245:83-94
22. Soto-Gutiérrez A N-AN, Zhao D, Rivas-Carrillo JD, Lebkowski J, Tanaka N, Fox IJ, Kobayashi N. Differentiation of mouse embryonic stem cells to hepatocyte-like cells by co-culture with human liver nonparenchymal cell lines. *Nat Protoc.* . 2007;2:347-356
23. Anna M. Wobus GK, Jin Shan, Marie-Cecile Wellner,, Jurgen Rohwedel JG, Bernd Fleischmann, Hugo A. Katus, Franz JHW-M. Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. *J Mol Cell Cardiol*. 1997;29:1525-1539
24. Martin-Bermudo MD. Integrins modulate the egfr signaling pathway to regulate tendon cell differentiation in the drosophila embryo. *Development 127*. 2000:2607-2615
25. YOHEI HAYASHI MKF, TETSUJI OKAMOTO, KIYOSHI OHNUMA, YASUFUMI MYOISHI,, YASUAKI FUKUHARA TA, J. DENRY SATO, RYU-ICHIRO HATA, MAKOTO ASASHIMA. Integrins regulate mouse embryonic stem cell self-renewal. *STEM CELLS*. 2007:3005–3015
26. Jurgen Rohwedel KG, Werner Zuschratter,, Shan Jin GA-H, Dieter Furst,, Reinhard Fassler AMW. Loss of b1 integrin function results in a retardation of myogenic, but an acceleration of neuronal, differentiation of embryonic stem cells in vitro. *Genomes & Developmental Control*. 1998:167–184
27. Qiao Zhou JB, Andrew Kanarek, Jayaraj Rajagopal & Douglas A. Melton. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. *Nature*. 2008;Vol 455:628
28. Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, Wernig M. Direct conversion of fibroblasts to functional neurons by defined factors. *Nature*. 2010;463:1035-1041
29. Szabo E, Rampalli S, Risueno RM, Schnerch A, Mitchell R, Fiebig-Comyn A, Levadoux-Martin M, Bhatia M. Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature*. 2010;468:521-526
30. Martin-Puig S, Wang Z, Chien KR. Lives of a heart cell: Tracing the origins of cardiac progenitors. *Cell stem cell*. 2008;2:320-331
31. Anna M. Wobus GW, Jiirgen Hescheler. Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses

to adrenergic and cholinergic agents and ca2 + channel blockers. *Differentiation*. 1991:173-182

32. Izhak Kehat DK-K, Mirit Snir, Hana Segev, Michal Amit,Amira Gepstein, Erella Livne, Ofer Binah, Joseph Itskovitz-Eldor, and Lior Gepstein. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J. Clin. Invest.* . 2001:407-414
33. Zwi-Dantsis L, Huber I, Habib M, Winterstern A, Gepstein A, Arbel G, Gepstein L. Derivation and cardiomyocyte differentiation of induced pluripotent stem cells from heart failure patients. *European Heart Journal*.
34. David A Elliott SRB, Katerina Koutsis, Elizabeth S Ng, Robert Jenny, Ebba L Lagerqvist, Christine Biben, Tanya Hatzistavrou, Claire E Hirst, Qing C Yu, Rhys J P Skelton, Dorien Ward-van Oostwaard, Sue Mei Lim, Ouda Khammy, Xueling Li, Susan, M Hawes, Richard P Davis, Adam L Goulburn, Robert Passier, Owen W J Prall, John M Haynes, Colin W Pouton, David M Kaye, Christine L Mummery, Andrew G Elefanty & Edouard G Stanley. Nkx2-5egfp/w hescescescs for isolation of human cardiac progenitors and cardiomyocytes. *Nat Protoc.* 2011;8
35. Kattman SJ, Witty AD, Gagliardi M, Dubois NC, Niapour M, Hotta A, Ellis J, Keller G. Stage-specific optimization of activin/nodal and bmp signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell*. 2011;8:228-240
36. Burridge Paul W, Keller G, Gold Joseph D, Wu Joseph C. Production of de novo cardiomyocytes: Human pluripotent stem cell differentiation and direct reprogramming. *Cell stem cell*.10:16-28
37. Noseda M, Peterkin T, Simoes FC, Patient R, Schneider MD. Cardiopoietic factors. *Circulation Research*. 2011;108:129-152
38. zur Nieden NI, Price FD, Davis LA, Everitt RE, Rancourt DE. Gene profiling on mixed embryonic stem cell populations reveals a biphasic role for {beta}-catenin in osteogenic differentiation. *Mol Endocrinol*. 2007;21:674-685
39. Ueno S, Weidinger G, Osugi T, Kohn AD, Golob JL, Pabon L, Reinecke H, Moon RT, Murry CE. Biphasic role for wnt/î²-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proceedings of the National Academy of Sciences*. 2007;104:9685-9690
40. Atsuhiko T. Naito IS, Hiroshi Akazawa, Kyoko Hidaka, Takayuki Morisaki, Akira Kikuchi,, Komuro I. Developmental stage-specific biphasic roles of wnt/b-catenin signaling in cardiomyogenesis and hematopoiesis. *PNAS*. 2006;103:19812-19817
41. THANH H. TRAN XW, CAROL BROWNE, YUNYU ZHANG, MARTINA SCHINKE, SEIGO IZUMO,MARK BURCIN. Wnt3a-induced mesoderm formation and cardiomyogenesis in human embryonic stem cells. *Stem Cell International*. 2009;27:1869
42. Passier R, Oostwaard DW-v, Snapper J, Kloots J, Hassink RJ, Kuijk E, Roelen B, de la Riviere AB, Mummery C. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. *Stem Cells*. 2005;23:772-780
43. Laflamme MA, Murry CE. Regenerating the heart. *Nat Biotech*. 2005;23:845-856
44. Takeuchi JK, Bruneau BG. Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors. *Nature*. 2009;459:708-711
45. Efe JA, Hilcove S, Kim J, Zhou H, Ouyang K, Wang G, Chen J, Ding S. Conversion of mouse fibroblasts into cardiomyocytes using a direct reprogramming strategy. *Nat Cell Biol*. 2011;13:215-222

46. Wang H, Cao, Nan., Spencer, Ian., Nie,Baoming., Ma, Tianhua., Xu, Tao., Zhang, Yu., Wang, Xiaojing., Srivastava, Deepak., Ding, Sheng. Small molecules enable cardiac reprogramming of mouse fibroblasts with a single factor, oct4. *Cell Reports*. 2014:1–10
47. Qian L, Huang Y, Spencer CI, Foley A, Vedantham V, Liu L, Conway SJ, Fu J-d, Srivastava D. In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. *Nature*. 2012;advance online publication
48. Laugwitz K-L, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, Lin L-Z, Cai C-L, Lu MM, Reth M, Platoshyn O, Yuan JXJ, Evans S, Chien KR. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature*. 2005;433:647-653
49. Moretti A, Lam J, Evans S, Laugwitz K. Biology of isl1+ cardiac progenitor cells in development and disease. *Cellular and Molecular Life Sciences (CMLS)*. 2007;64:674 – 682
50. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell*. 2003;114:763-776
51. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, Pocius J, Michael LH, Behringer RR, Garry DJ, Entman ML, Schneider MD. Cardiac progenitor cells from adult myocardium: Homing, differentiation, and fusion after infarction. *Proceedings of the National Academy of Sciences*. 2003;100:12313-12318
52. Daniel CMMAPMSMRTJHJARSBSCGTDG. Persistent expression of the atp-binding cassette transporter, abcg2, identifies cardiac sp cells in the developing and adult heart. *Genomes & Developmental Control*. 2004;265:275
53. Elisa Messina LDA, Giacomo Frati, Stefania Morrone, Stefano Chimenti, Fabio Fiordaliso, Monica Salio, Massimo Battaglia, Michael V.G. Latronico, Marcello Coletta, Elisabetta Vivarelli, Luigi Frati, Giulio Cossu and Alessandro Giacomello. Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res.* 2004;95:911-921
54. Moretti A, Caron L, Nakano A, Lam JT, Bernshausen A, Chen Y, Qyang Y, Bu L, Sasaki M, Martin-Puig S, Sun Y, Evans SM, Laugwitz K-L, Chien KR. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell*. 2006;127:1151
55. Cai C-L, Liang X, Shi Y, Chu P-H, Pfaff SL, Chen J, Evans S. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. *Developmental cell*. 2003;5:877-889
56. Thomas Brade LSP, Alessandra Moretti, Kenneth R. Chien, Karl-Ludwing Lauwitz. Embryonic heart progenitors and cardiogenesis. *Cold spring Harb Perspect Med*. 2013;3:1-13
57. Arnold SJR, Elizabeth J. . Making a commitment- cell lineage allocation and axis patterning in the early mouse embryo. *Nature Reviews Molecular Cell Biology* 2009;10
58. Patrick P. L. Tam DAFL. Gene function in mouse embryogenesis: Get set for gastrulation. *Nature*. 2007;8
59. Abu-Issa R, Kirby ML. Heart field: From mesoderm to heart tube. *Annual Review of Cell and Developmental Biology*. 2007;23:45-68

60. Zhou B, Ma Q, Rajagopal S, Wu SM, Domian I, Rivera-Feliciano J, Jiang D, von Gise A, Ikeda S, Chien KR, Pu WT. Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature*. 2008
61. Francesca Rochais KMaRGK. Signaling pathways controlling second heart field development. *Circulation Research*. 2009;104:933-942
62. Vincent SD, Buckingham ME, Peter K. How to make a heart: The origin and regulation of cardiac progenitor cells. *Current topics in developmental biology*. Academic Press; 2010:1-41.
63. Cohen ED, Wang Z, Lepore JJ, Lu MM, Taketo MM, Epstein DJ, Morrisey EE. Wnt/î²-catenin signaling promotes expansion of isl-1â€"positive cardiac progenitor cells through regulation of fgf signaling. *The Journal of Clinical Investigation*. 2007;117:1794-1804
64. Qyang Y, Martin-Puig S, Chiravuri M, Chen S, Xu H, Bu L, Jiang X, Lin L, Granger A, Moretti A, Caron L, Wu X, Clarke J, Taketo MM, Laugwitz K-L, Moon RT, Gruber P, Evans SM, Ding S, Chien KR. The renewal and differentiation of isl1+ cardiovascular progenitors are controlled by a wnt/β-catenin pathway. *Cell Stem Cell*. 2007;1:165-179
65. Federica Limana MCC, Antonia Germani. The epicardium in cardiac repair: From the stem cell view. *Pharmacology & Therapeutics* 2011;129:82-96
66. Cai C-L, Martin JC, Sun Y, Cui L, Wang L, Ouyang K, Yang L, Bu L, Liang X, Zhang X, Stallcup WB, Denton CP, McCulloch A, Chen J, Evans SM. A myocardial lineage derives from tbx18 epicardial cells. *Nature*. 2008;454:104-108
67. Katz TC SM, Degenhardt K, Rivera-Feliciano J, Johnson RL, Epstein JA, Tabin CJ. Distinct compartments of the proepicardial organ give rise to coronary vascular endothelial cells. *Dev Cell.* . 2012 22:639-650
68. Zhou B, Gise Av, Ma Q, Rivera-Feliciano J, Pu WT. Nkx2-5- and isl1-expressing cardiac progenitors contribute to proepicardium. *Biochemical and Biophysical Research Communications*. 2008;375:450-453
69. van Wijk B, van den Hoff M. Epicardium and myocardium originate from a common cardiogenic precursor pool. *Trends in Cardiovascular Medicine*. 2010;20:1-7
70. Red-Horse K, Ueno H, Weissman IL, Krasnow MA. Coronary arteries form by developmental reprogramming of venous cells. *Nature*. 2010;464:549-553
71. Snider P, Olaopa M, Firulli AB, Conway SJ. Cardiovascular development and the colonizing cardiac neural crest lineage. *ScientificWorldJournal*. 2007;7:1090–1113
72. Stoller JZ, Epstein JA. Cardiac neural crest. *Seminars in Cell & Developmental Biology*. 2005;16:704-715
73. Anna Keyte MRH. The neuralcrestincardiaccongenitalanomalies. *Differentiation*. 2012;84:25-40
74. Song L, Li Y, Wang K, Zhou CJ. Cardiac neural crest and outflow tract defects in lrp6 mutant mice. *Developmental Dynamics*. 2010;239:200-210
75. Tan CC, Sindhu KV, Li S, Nishio H, Stoller JZ, Oishi K, Puttreddy S, Lee TJ, Epstein JA, Walsh MJ, Gelb BD. Transcription factor ap2{delta} associates with ash2l and alr, a trithorax family histone methyltransferase, to activate hoxc8 transcription. *Proceedings of the National Academy of Sciences*. 2008;105:7472-7477
76. Bamforth SD, Braganca J, Eloranta JJ, Murdoch JN, Marques FIR, Kranc KR, Farza H, Henderson DJ, Hurst HC, Bhattacharya S. Cardiac malformations, adrenal

agenesis, neural crest defects and exencephaly in mice lacking cited2, a new tfap2 co-activator. *Nat. Genet.* 2001;29:469-474
77. Dunwoodie SL, Rodriguez TA, Beddington RSP. Msg1 and mrg1, founding members of a gene family, show distinct patterns of gene expression during mouse embryogenesis. *Mechanisms of Development*. 1998;72:27
78. Toshihiro Shioda MHF, Kurt J. Isselbacher. Msgl, a novel melanocyte-specific gene, encodes a nuclear protein and is associated with pigmentation. *Proc. Natl. Acad. Sci. USA*. 1996;93:12298-12303
79. Bhattacharya S, Michels CL, Leung MK, Arany ZP, Kung AL, Livingston DM. Functional role of p35srj, a novel p300/cbp binding protein, during transactivation by hif-1. *Genes Dev.* 1999;13:64-75
80. Sun HB, Zhu YX, Yin T, Sledge G, Yang Y-C. Mrg1, the product of a melanocyte-specific gene related gene, is a cytokine-inducible transcription factor with transformation activity. *Proceedings of the National Academy of Sciences of the United States of America*. 1998;95:13555-13560
81. Leung M-K, Jones T, Michels CL, Livingston DM, Bhattacharya S. Molecular cloning and chromosomal localization of the human cited2 gene encoding p35srj/mrg1. *Genomics*. 1999;61:307-313
82. Jane E. Andrews MJON, Michele Binder, Toshi Shioda, Andrew H. Sinclair. Isolation and expression of a novel member of the cited family. *Mechanisms of Development* 2000:305-308
83. Braganca J, Swingler T, Marques FIR, Jones T, Eloranta JJ, Hurst HC, Shioda T, Bhattacharya S. Human creb-binding protein/p300-interacting transactivator with ed-rich tail (cited) 4, a new member of the cited family, functions as a co-activator for transcription factor ap-2. *J. Biol. Chem.* 2002;277:8559-8565
84. Chan HM, La Thangue NB. P300/cbp proteins: Hats for transcriptional bridges and scaffolds. *J Cell Sci*. 2001;114:2363-2373
85. Grossman SR, Deato ME, Brignone C, Chan HM, Kung AL, Tagami H, Nakatani Y, Livingston DM. Polyubiquitination of p53 by a ubiquitin ligase activity of p300. *Science*. 2003;300:342-344
86. Toshi Shioda MHF, Kurt J. Isselbacher. Msg1 and its related protein mrg1 share a transcription activating domain. *Gene*. 1997;204:235–241
87. Sperling S, Grimm CH, Dunkel I, Mebus S, Sperling H-P, Ebner A, Galli R, Lehrach H, Fusch C, Berger F, Hammer S. Identification and functional analysis of cited2 mutations in patients with congenital heart defects. *Human Mutation*. 2005;26:575-582
88. Chiann-mun Che. JB, Catherine Cosgrove, Jose Braganca, Ana Cuenda, Simon D. Bamforth, Jurgen E. Schneider, Hugh Watkins, Bernard Keavney, Benjamin Davies, Shoumo Bhattacharya. Functional significance of srj domain mutations in cited2. 2012
89. Min Xu XW, Yonggang Li, Xiaofei Yang, Jihua Hu, Min Zheng, Jie Tian. Cited2 mutation and methylation in children with congenital heart disease. *Journal of Biomedical Science*. 2014;21:7
90. Yokota H, Goldring MB, Sun HB. Cited2-mediated regulation of mmp-1 and mmp-13 in human chondrocytes under flow shear. *Journal of Biological Chemistry*. 2003;278:47275-47280
91. Yin Z, Haynie J, Yang X, Han B, Kiatchoosakun S, Restivo J, Yuan S, Prabhakar NR, Herrup K, Conlon RA, Hoit BD, Watanabe M, Yang Y-C. The essential role of

cited2, a negative regulator for hif-1alpha , in heart development and neurulation. *Proc. Natl Acad. of Sci. U.S.A*. 2002;99:10488-10493
92. Tien ES, Davis JW, Vanden Heuvel JP. Identification of the creb-binding protein/p300-interacting protein cited2 as a peroxisome proliferator-activated receptor alpha coregulator. *J. Biol. Chem.* 2004;279:24053-24063
93. Glenn DJ, Maurer RA. Mrg1 binds to the lim domain of lhx2 and may function as a coactivator to stimulate glycoprotein hormone alpha -subunit gene expression. *J. Biol. Chem.* 1999;274:36159-36167
94. Bamforth SD, Braganca J, Farthing CR, Schneider JE, Broadbent C, Michell AC, Clarke K, Neubauer S, Norris D, Brown NA, Anderson RH, Bhattacharya S. Cited2 controls left-right patterning and heart development through a nodal-pitx2c pathway. *Nat. Genet.* 2004;36:1189-1196
95. Fox SB, Braganca J, Turley H, Campo L, Han C, Gatter KC, Bhattacharya S, Harris AL. Cited4 inhibits hypoxia-activated transcription in cancer cells, and its cytoplasmic location in breast cancer is associated with elevated expression of tumor cell hypoxia-inducible factor 1{alpha}. *Cancer Res*. 2004;64:6075-6081
96. Yahata T, Takedatsu H, Dunwoodie SL, Braganca J, Swingler T, Withington SL, Hur J, Coser KR, Isselbacher KJ, Bhattacharya S, Shioda T. Cloning of mouse cited4, a member of the cited family p300/cbp-binding transcriptional coactivators: Induced expression in mammary epithelial cells. *Genomics*. 2002;80:601-613
97. Boström P, Mann N, Wu J, Quintero PA, Plovie ER, Panáková D, Gupta RK, Xiao C, MacRae CA, Rosenzweig A, Spiegelman BM. C/ebp[beta] controls exercise-induced cardiac growth and protects against pathological cardiac remodeling. *Cell*. 2010;143:1072-1083
98. Chou Y-T, Yang Y-C. Post-transcriptional control of cited2 by transforming growth factor beta: Regulation via smads and cited2 coding region. *J. Biol. Chem.* 2006;281:18451-18462
99. Pouponnot C, Jayaraman L, Massague J. Physical and functional interaction of smads and p300/cbp. *J. Biol. Chem.* 1998;273:22865-22868
100. Chou YT, Wang H, Chen Y, Danielpour D, Yang YC. Cited2 modulates tgf-beta-mediated upregulation of mmp9. *Oncogene*. 2006;25:5547-5560
101. Pritsker M, Ford NR, Jenq HT, Lemischka IR. Genomewide gain-of-function genetic screen identifies functionally active genes in mouse embryonic stem cells. *Proc. Natl Acad. of Sci. U.S.A*. 2006;103:6946-6951
102. Chen C-m, Bentham J, Cosgrove C, Braganca J, Cuenda A, Bamforth SD, Schneider JE, Watkins H, Keavney B, Davies B, Bhattacharya S. Functional significance of srj domain mutations in cited2. *PLoS ONE*. 2012;7:e46256
103. Gabut M, Samavarchi-Tehrani P, Wang X, Slobodeniuc V, O'Hanlon D, Sung H-K, Alvarez M, Talukder S, Pan Q, Mazzoni Esteban O, Nedelec S, Wichterle H, Woltjen K, Hughes Timothy R, Zandstra PW, Nagy A, Wrana Jeffrey L, Blencowe Benjamin J. An alternative splicing switch regulates embryonic stem cell pluripotency and reprogramming. *Cell*. 2011
104. Du J, Chen Y, Li Q, Han X, Cheng C, Wang Z, Danielpour D, Dunwoodie SL, Bunting KD, Yang Y-C. Hif-1a deletion partially rescues defects of hematopoietic stem cell quiescence caused by cited2 deficiency. *Blood*. 2012;119:2789-2798
105. Kranc KR, Schepers H, Rodrigues NP, Bamforth S, Villadsen E, Ferry H, Bouriez-Jones T, Sigvardsson M, Bhattacharya S, Eirik Jacobsen S, Enver T. Cited2 is an

essential regulator of adult hematopoietic stem cells. *Cell Stem Cell*. 2009;5:659-665

106. Li Q, Ramirez-Bergeron DL, Dunwoodie SL, Yang Y-C. Cited2 controls pluripotency and cardiomyocyte differentiation of murine embryonic stem cells through oct4. *Journal of Biological Chemistry*. 2012
107. Withington SL, Scott AN, Saunders DN, Lopes Floro K, Preis JI, Michalicek J, Maclean K, Sparrow DB, Barbera JPM, Dunwoodie SL. Loss of cited2 affects trophoblast formation and vascularization of the mouse placenta. *Dev. Biol.* 2006;294:67-82
108. Chen Y, Doughman Y-q, Gu S, Jarrell A, Aota S-i, Cvekl A, Watanabe M, Dunwoodie SL, Johnson RS, van Heyningen V, Kleinjan DA, Beebe DC, Yang Y-C. Cited2 is required for the proper formation of the hyaloid vasculature and for lens morphogenesis. *Development*. 2008:135(117):2939-2948
109. Chen Y, Haviernik P, Bunting KD, Yang Y-C. Cited2 is required for normal hematopoiesis in the murine fetal liver. *Blood*. 2007;110:2889-2898
110. Barbera JPM, Rodriguez TA, Greene NDE, Weninger WJ, Simeone A, Copp AJ, Beddington RSP, Dunwoodie S. Folic acid prevents exencephaly in cited2 deficient mice. *Hum. Mol. Genet.* 2002;11:283-293
111. Weninger WJ, Floro KL, Bennett MB, Withington SL, Preis JI, Barbera JPM, Mohun TJ, Dunwoodie SL. Cited2 is required both for heart morphogenesis and establishment of the left-right axis in mouse development. *Development*. 2005;132:1337-1348
112. MacDonald ST, Bamforth SD, Chen C-M, Farthing CR, Franklyn A, Broadbent C, Schneider JE, Saga Y, Lewandoski M, Bhattacharya S. Epiblastic cited2 deficiency results in cardiac phenotypic heterogeneity and provides a mechanism for haploinsufficiency. *Cardiovasc Res*. 2008:cvn101
113. Feil R, Wagner Jr, Metzger D, Chambon P. Regulation of cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochemical and Biophysical Research Communications*. 1997;237:752-757
114. Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. Functional expression cloning of nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell*. 2003;113:643-655
115. Domian IJ, Chiravuri M, van der Meer P, Feinberg AW, Shi X, Shao Y, Wu SM, Parker KK, Chien KR. Generation of functional ventricular heart muscle from mouse ventricular progenitor cells. *Science*. 2009;326:426-429
116. Jian R, Cheng X, Jiang J, Deng S, Hu F, Zhang J. A cdna-based random rna interference library for functional genetic screens in embryonic stem cells. *Stem Cells*. 2007;25:1904-1912
117. Kranc KR, Bamforth SD, Braganca J, Norbury C, van Lohuizen M, Bhattacharya S. Transcriptional coactivator cited2 induces bmi1 and mel18 and controls fibroblast proliferation via ink4a/arf. *Mol. Cell. Biol.* 2003;23:7658-7666
118. Sachinidis A, Schwengberg S H-AR, Mariappan D, Kamisetti N, Seelig B, Berkessel A, Hescheler J. Identification of small signalling molecules promoting cardiac-specific differentiation of mouse embryonic stem cells. *Cell Physiol Biochem.* 2006;18:303-314
119. Orimo T, Taga M, Matsui H, Minaguchi H. The effect of activin-a on the development of mouse preimplantation embryos in vitro. *Journal of Assisted Reproduction and Genetics*. 1996;13:669-674

120. Gouon-Evans V, Boussemart L, Gadue P, Nierhoff D, Koehler CI, Kubo A, Shafritz DA, Keller G. Bmp-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. *Nat Biotech*. 2006;24:1402
121. Ivanova N, Dobrin R, Lu R, Kotenko I, Levorse J, DeCoste C, Schafer X, Lun Y, Lemischka IR. Dissecting self-renewal in stem cells with rna interference. *Nature*. 2006;442:533-538
122. Lozano-Velasco E, Chinchilla A, Martínez-Fernández S, Hernández-Torres F, Navarro F, Lyons GE, Franco D, Aránega AE. Pitx2c modulates cardiac-specific transcription factors networks in differentiating cardiomyocytes from murine embryonic stem cells. *Cells Tissues Organs*. 2011;Epub ahead of print
123. Bondue A, Lapouge G, Paulissen C, Semeraro C, Lacovino M, Kyba M, Blanpain C. Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. *Cell Stem Cell*. 2008;3:69-84
124. Zhong X, Jin Y. Critical roles of coactivator p300 in mouse embryonic stem cell differentiation and nanog expression. *J. Biol. Chem.* 2009;284:9168-9175
125. Ogawa K, Saito A, Matsui H, Suzuki H, Ohtsuka S, Shimosato D, Morishita Y, Watabe T, Niwa H, Miyazono K. Activin-nodal signaling is involved in propagation of mouse embryonic stem cells. *J. Cell. Sci.* 2007;120:55-65
126. Martínez-Fernandez S, Hernández-Torres F, Franco D, Lyons GE, Navarro F, Aránega AE. Pitx2c overexpression promotes cell proliferation and arrests differentiation in myoblasts. *Developmental Dynamics*. 2006;235:2930-2939
127. Fujimori H, Asahina K, Shimizu-Saito K, Ikeda R, Tanaka Y, Teramoto K, Morita I, Teraoka H. Vascular endothelial growth factor promotes proliferation and function of hepatocyte-like cells in embryoid bodies formed from mouse embryonic stem cells. *Journal of Hepatology*. 2008;48:962-973
128. Snyder M, Huang X-Y, Zhang JJ. Stat3 directly controls the expression of tbx5, nkx2.5, and gata4 and is essential for cardiomyocyte differentiation of p19cl6 cells. *Journal of Biological Chemistry*. 2010;285:23639-22646
129. Nimura K, Ura K, Shiratori H, Ikawa M, Okabe M, Schwartz RJ, Kaneda Y. A histone h3 lysine 36 trimethyltransferase links nkx2-5 to wolf-hirschhorn syndrome. *Nature*. 2009;460
130. Patrick Van Vliet SMW, Stephane Zaffran and Michel Puceat. Early cardiac development: A view from stem cells to embryos. *Cardiovascular Research*. 2012
131. Zhu Y, Gramolini AO, Walsh MA, Zhou Y-Q, Slorach C, Friedberg MK, Takeuchi JK, Sun H, Henkelman RM, Backx PH, Redington AN, MacLennan DH, Bruneau BG. Tbx5-dependent pathway regulating diastolic function in congenital heart disease. *Proceedings of the National Academy of Sciences*. 2008;105:5519-5524
132. Habets PEMH, Moorman AFM, Clout DEW, van Roon MA, Lingbeek M, van Lohuizen M, Campione M, Christoffels VM. Cooperative action of tbx2 and nkx2.5 inhibits anf expression in the atrioventricular canal: Implications for cardiac chamber formation. *Genes & Development*. 2002;16:1234-1246
133. Goetz SC BD, Conlon FL. . Tbx5 is required for embryonic cardiac cell cycle progression. *Development*. 2006:2575-2584
134. Niakan KK, Ji H, Maehr R, Vokes SA, Rodolfa KT, Sherwood RI, Yamaki M, Dimos JT, Chen AE, Melton DA, McMahon AP, Eggan K. Sox17 promotes differentiation in mouse embryonic stem cells by directly regulating extraembryonic gene expression and indirectly antagonizing self-renewal. *Genes & Development*. 2010;24:312-326

135. Liu Y, Asakura M, Inoue H, Nakamura T, Sano M, Niu Z, Chen M, Schwartz RJ, Schneider MD. Sox17 is essential for the specification of cardiac mesoderm in embryonic stem cells. *Proceedings of the National Academy of Sciences*. 2007;104:3859–3864
136. Costello I, Pimeisl I-M, Drager S, Bikoff EK, Robertson EJ, Arnold SJ. The t-box transcription factor eomesodermin acts upstream of mesp1 to specify cardiac mesoderm during mouse gastrulation. *Nat Cell Biol*. 2011;13:1084-1091
137. David R, Jarsch VB, Schwarz F, Nathan P, Gegg M, Lickert H, Franz W-M. Induction of mesp1 by brachyury(t) generates the common multipotent cardiovascular stem cell. *Cardiovascular Research*. 2011;92:115-122
138. Yumiko Saga SM-T, Atsuya Takagi, Satoshi Kitajima, Jun-ichi Miyazaki, Tohru Inoue. Mesp1 is expressed in the heart precursor cells and required for the formation of a single heart tube. *Development* 1999;126:3437-3447
139. Lindsley R, Gill J, Murphy T, Langer E, Cai M, Mashayekhi M, Wang W, Niwa N, Nerbonne J, Kyba M, Murphy K. Mesp1 coordinately regulates cardiovascular fate restriction and epithelial-mesenchymal transition in differentiating escs. *Cell Stem Cell*. 2008;3:55-68
140. Saga Y, Kitajima, S., Miyagawa-Tomita, S. Mesp1 expression is the earliest sign of cardiovascular development. *Trends Cardiovasc Med* 2000;10:345–352
141. Tada S, Era T, Furusawa C, Sakurai H, Nishikawa S, Kinoshita M, Nakao K, Chiba T, Nishikawa S-I. Characterization of mesendoderm: A diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. *Development*. 2005;132:4363-4374
142. Kitamura R, Takahashi T, Nakajima N, Isodono K, Asada S, Ueno H, Ueyama T, Yoshikawa T, Matsubara H, Oh H. Stage-specific role of endogenous smad2 activation in cardiomyogenesis of embryonic stem cells. *Circ Res*. 2007;101:78-87
143. Risebro CA, Smart N, Dupays N, Breckenridge R, Mohun TJ, Riley PR. Hand1 regulates cardiomyocyte proliferation versus differentiation in the developing heart *Development*. 2006;133:4595-4606
144. Molkentin JD. The zinc finger-containing transcription factors gata-4, -5, and -6. *Journal of Biological Chemistry*. 2000;275:38949-38952
145. Roong Zhao AJW, Michele A. Battle, Jixuan Li, Benjamin J. Bondow, Stephen A. Duncan. Loss of both gata4 and gata6 blocks cardiac myocyte differentiation and results in acardia in mice. *Dev Biol.* 2008;317: 614–619
146. Dufort D, Schwartz L, Harpal K, Rossant J. The transcription factor hnf3b is required in visceral endoderm for normal primitive streak morphogenesis. *Development*. 1998;125:3015-3025
147. Soudais C, Bielinska M, Heikinheimo M, MacArthur CA, Narita N, Saffitz JE, Simon MC, Leiden JM, Wilson DB. Targeted mutagenesis of the transcription factor gata-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation in vitro. *Development*. 1995;121:3877-3888
148. Kanda S, Shiroi A, Ouji Y, Birumachi J, Ueda S, Fukui H, Tatsumi K, Ishizaka S, Takahashi Y, Yoshikawa M. In vitro differentiation of hepatocyte-like cells from embryonic stem cells promoted by gene transfer of hepatocyte nuclear factor 3 beta. *Hepatol. Res.* 2003;26:225
149. Baba S, Heike T, Yoshimoto M, Umeda K, Doi H, Iwasa T, Lin X, Matsuoka S, Komeda M, Nakahata T. Flk1+ cardiac stem/progenitor cells derived from

embryonic stem cells improve cardiac function in a dilated cardiomyopathy mouse model. *Cardiovasc Res*. 2007;76:119-131

150. MacDonald ST, Bamforth SD, BraganÃ§a J, Chen C-M, Broadbent C, Schneider JrE, Schwartz RJ, Bhattacharya S. A cell-autonomous role of cited2 in controlling myocardial and coronary vascular development. *European Heart Journal*. 2012
151. Stefan Irion RLC, Hervé Luche, Injune Kim, Sean J. Morrison, Hans-Joerg Fehling, Keller GM. Temporal specification of blood progenitors from mouse embryonic stem cells and induced pluripotent stem cells. *Development*. 2010;137:2829-2839
152. Zaffran S, Frasch M. Early signals in cardiac development. *Circ Res*. 2002;91:457-469
153. Silvia Martin-Puig ZW, Kenneth R. Chien. Lives of a heart cell: tracing the origins of cardiac progenitors. *Cell Stem Cell*. 2008;2:12
154. Loez-Sanchez C, Garcia-Martinez V. Molecular determinants of cardiac specification. *Cardiovascular Research*. 2011
155. Ma Q, Zhou B, Pu WT. Reassessment of isl1 and nkx2-5 cardiac fate maps using a gata4-based reporter of cre activity. *Developmental Biology*. 2008;323:98-104
156. Qyang Y, Martin-Puig S, Chiravuri M, Chen S, Xu H, Bu L, Jiang X, Lin L, Granger A, Moretti A, Caron L, Wu X, Clarke J, Taketo M, Laugwitz K, Moon R, Gruber P, Evans S, Ding S, Chien K. The renewal and differentiation of isl1+ cardiovascular progenitors are controlled by a wnt/beta-catenin pathway. *Cell Stem Cell*. 2007;1:165-179
157. Klaus A, MÃ¼ller M, Schulz H, Saga Y, Martin JF, Birchmeier W. Wnt/î²-catenin and bmp signals control distinct sets of transcription factors in cardiac progenitor cells. *Proceedings of the National Academy of Sciences*.
158. Dodou E, Verzi MP, Anderson JP, Xu S-M, Black BL. Mef2c is a direct transcriptional target of isl1 and gata factors in the anterior heart field during mouse embryonic development. *Development*. 2004;131:3931-3942
159. Pingzhu Zhou AH, William T. Pu. Regulation of gata4 transcriptional activity in cardiovascular development and disease. *Current Topics in Developmental Biology*. 2012;100
160. Chulan Kwon LQPC, Vishal Nigam, Josh Arnold, Deepak Srivastava. A regulatory pathway involving notch1/β-catenin/isl1 determines cardiac progenitor cell fate. *NHI public access*. 2009;11:951-957
161. Kerppola TK. Visualization of molecular interactions using bimolecular fluorescence complementation analysis: Characteristics of protein fragment complementation. *Chemical Society Reviews*. 2009;38:2876-2886
162. Lee O-H, Kim H, He Q, Baek HJ, Yang D, Chen L-Y, Liang J, Chae HK, Safari A, Liu D, Songyang Z. Genome-wide yfp fluorescence complementation screen identifies new regulators for telomere signaling in human cells. *Molecular & Cellular Proteomics*. 2011;10
163. Wu Z, Zhang W, Chen G, Cheng L, Liao J, Jia N, Gao Y, Dai H, Yuan J, Cheng L, Xiao L. Combinatorial signals of activin/nodal and bone morphogenic protein regulate the early lineage segregation of human embryonic stem cells. *J. Biol. Chem.* 2008;283:24991-25002
164. N. Ray Dunn SDV, Leif Oxburgh, Elizabeth J. Robertson* and Elizabeth K. Bikoff. Combinatorial activities of smad2 and smad3 regulate mesoderm formation and patterning in the mouse embryo. *Development*. 2004;131:1717-1728

165. Besser D. Expression of nodal, lefty-a, and lefty-b in undifferentiated human embryonic stem cells requires activation of smad2/3. *J. Biol. Chem.* 2004;279:45076-45084
166. Fei T, Zhu S, Xia K, Zhang J, Li Z, Han J-DJ, Chen Y-G. Smad2 mediates activin/nodal signaling in mesendoderm differentiation of mouse embryonic stem cells. *Cell Res*. 2010;20:1306-1318
167. Dr. Kristina C. Pfendler CSC, Juanito J. Meneses, and Roger A. Pedersen. . Overexpression of nodal promotes differentiation of mouse embryonic stem cells into mesoderm and endoderm at the expense of neuroectoderm formation. *Stem Cells and Development*. 2005;14:162-172
168. Kwon C, Qian L, Cheng P, Nigam V, Arnold J, Srivastava D. A regulatory pathway involving notch1/β-catenin/isl1 determines cardiac progenitor cell fate. *Nat cell biol*. 2009;11:951-957
169. Kappen C, Salbaum JM. Identification of regulatory elements in the isl1 gene locus *Int. J. Dev. Biol*. 2009;53:935 - 946
170. Aibin He SWK, Qing Ma, William T. Pu,. Co-occupancy by multiple cardiac transcription factors identifies transcriptional enhancers active in heart. *PNAS*. 2011;108:5632-5637
171. Klaus A, Saga Y, Taketo MM, Tzahor E, Birchmeier W. Distinct roles of wnt/î²-catenin and bmp signaling during early cardiogenesis. *Proceedings of the National Academy of Sciences*. 2007;104:18531-18536
172. Kidder BL, Palmer S. Examination of transcriptional networks reveals an important role for tcfap2c, smarca4, and eomes in trophoblast stem cell maintenance. *Genome Research*. 2010;20:458-472
173. Christoffels VM, Moorman AFM. Development of the cardiac conduction system. *Circulation: Arrhythmia and Electrophysiology*. 2009;2:195-207
174. Hagen R. Witzel BJ, Chong Pyo Choe, J. Gage Crump, Thomas Braun,Gergana Dobreva. The lim protein ajuba restricts the second heart field progenitor pool by regulating isl1 activity. *Developmental Cell* 2012;23:58-70
175. Michell AC, Bragança J, Broadbent C, Joyce B, Franklyn A, Schneider JE, Bhattacharya S, Bamforth SD. A novel role for transcription factor lmo4 in thymus development through genetic interaction with cited2. *Developmental Dynamics*. 2010;239:1988-1994
176. Li T LY, Jia ZQ, Chen P, Ma KT, Zhou CY. Carboxyl terminus of nkx2.5 impairs its interaction with p300. *J Mol Biol.* 2007;370:976-992
177. Alcmène Chalazonitis FDA, Tuan D. Pham, John A. Kessler, and Michael D. Gershon. Bone morphogenetic proteins regulate enteric gliogenesis by modulating erbb3 signaling. *Dev Biol.* 2011; 350:64–79