IDENTIFICATION AND CHARACTERIZATION OF CRIPTO ANTAGONISTS TO IMPROVE THE USE OF STEM CELL-BASED THERAPY IN NEURODEGENERATIVE DISORDERS

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1. SUMMARY

1.1 Identification and characterization of Cripto antagonists to improve the use of stem cell-based therapy in neurodegenerative disorders

Recent breakthroughs in stem cell research underscore the importance of controlling stem cell differentiation for the success of cell-based transplantation therapies in neurodegenerative diseases, such as Parkinson Disease (PD). In this regard, Embryonic Stem (ES) cells have been shown to serve as a source for deriving the optimal dopaminergic (DA) cells to restore the nigrostriatal system. However, our knowledge about the molecular mechanisms implicated in the determination of the neural fate in ES cells is still incomplete and thus their differentiation is poorly controlled. Recent findings from our laboratory indicate that the EGF-CFC protein Cripto is a key player in the signaling pathways that control neural differentiation of ES cells. Cripto is a GPI-anchored protein and a co-receptor for Nodal, a TGF-β family ligand. Cripto-dependent Nodal signaling is required for early embryogenesis; this signaling depends on the Activin type II serine/threonine kinase receptor (ActRII) and the Activin type I serine/threonine kinase receptor (Alk4), which, once activated, phosphorylate the downstream transcriptional coactivator Smad2. Cripto is strictly required for ES cell differentiation to cardiomyocytes and it negatively regulates neuronal differentiation of ES cells; indeed, disruption of *Cripto* in ES cells results in increased dopaminergic differentiation and reduced tumor formation, upon transplantation in rat models of PD. As an extracellular molecule, Cripto is a promising target for therapy. The specific aims of my projecy are: a) to identify molecules able to inhibit Cripto/receptor interaction by exploiting a powerful experimental approach based on the use of combinatorial chemistry; b) to improve the therapeutic potential of ES cells for cell replacement therapy in PD. We have thus exploited a novel experimental approach based on the use of combinatorial chemistry combined to ES cell differentiation. By using an ELISA-based assay, we previously identified a tetrameric tripeptide able to block efficiently the Cripto/ Alk4receptor interaction.

Our results show that this blocking peptides, by inhibiting the endogenous Cripto signaling, impairs cardiac differentiation and redirects the differentiation of ES cells to a neural fate. In addition, the Cripto/Alk4 Blocking peptide promotes ES cell differentiation to dopaminegic neurons.

All together our data provide evidence that, through the addition of small, non-toxic activators/inhibitors of signaling pathways, the differentiation of pluripotent ES cells might be controlled for the production of specific cell types, suitable for the use in animal model of human disease. Worth noting, the ability to direct ES cell fate solely through the use of extracellular factors, without the need to manipulate ES cells genetically, may permit a direct extension of this strategy to human cells.

1.2 Identificazione e caratterizzazione di antagonisti di Cripto per migliorare l'uso delle cellule staminali in malattie neurodegenerative

La riparazione di tessuti ed organi danneggiati è una necessità per la sopravvivenza di tutti gli individui. In questa prospettiva, il trapianto cellulare rappresenta una grande opportunità per sopperire all'incapacità di diversi tessuti dell'organismo adulto di sostituire le parti lese. Il successo di tale strategia è limitato dall'accesso alla preparazione ed alla disponibilità di cellule altamente specializzate nonchè alla non omogeneità dei tipi cellulari prodotti. In questo scenario, le cellule staminali embrionali (ES) rappresentano una sorgente ottimale per ottenere diversi tipi di cellule differenziate, grazie alla loro capacità di replicarsi un numero illimitato di volte e di dare origine a qualsiasi tipo cellulare specializzato dell'organismo adulto. Inoltre, tali cellule rappresentano un ottimo sistema modello per studiare i meccanismi molecolari che controllano le prime fasi dello sviluppo dei mammiferi e quindi i processi di specificazione e differenziamento dei diversi *lineages* cellulari. Tali meccanismi sono al momento ancora largamente sconosciuti e suscitano notevole interesse. Infatti, studi emergenti da differenti campi di ricerca fanno ipotizzare che le stesse molecole e meccanismi che operano durante lo sviluppo embrionale sono anche coinvolti in processi, sia fisiologici che patologici, attivi nell'adulto. Ad esempio, l'associazione tra embriogenesi e tumorigenesi è molto forte e sta aprendo nuove strade nella ricerca oncologica.

Studi recenti condotti sulle cellule staminali sottolineano l'importanza di controllare il differenziamento delle stesse per il successo della terapia cellulare in malattie neurodegenerative come il Parkinson [1]. È stato dimostrato, infatti, che le cellule ES, possono essere un'ottima risorsa per ottenere neuroni dopaminergici da utilizzare per il trapianto [2]. Tuttavia, l'impiego delle ES in approcci di medicina rigenerativa ha un grosso limite legato alla presenza di cellule indifferenziate che possono dar origine alla formazione e allo sviluppo di teratomi. Ne consegue, quindi, che la comprensione dei meccanismi molecolari alla base dei processi di specificazione e differenziamento dei diversi *lineages* cellulari nei mammiferi è fondamentale al fine di i) controllare il differenziamento delle cellule staminali a scopi terapeutici in approcci di medicina rigenerativa e ii) di identificare *pathways* di trasduzione del segnale attivi in processi biologici strettamente correlati come l'embriogenesi e la tumorigenesi.

In questo scenario, il gene *cripto* svolge un ruolo chiave. Cripto/TDGF-1 (teratocarcinoma-derived growth factor-1) è il capostipite della famiglia delle proteine extracellulari EGF-CFC, caratteristiche dei vertebrati, a cui appartengono FRL-1 in Xenopus, oep (one-eyed-pinhead) in Zebrafish, Cripto e cryptic nel topo e nell'uomo [3]. Tutte le proteine della famiglia EGF-CFC mostrano caratteristiche strutturali comuni: una regione idrofobica all'estremità N-terminale che ne media la secrezione, una regione EGF-simile, una regione CFC (da *cripto, FRL-1, cryptic*) caratterizzata da sei cisteine la cui spaziatura è conservata ed infine una piccola regione idrofobica all'estremità C-terminale.

Cripto è espresso sia nelle cellule ES che durante le fasi precoci dello sviluppo embrionale, mentre nell'adulto la sua espressione è riattivata in diversi tumori quali il

carcinoma del colon, del pancreas, ovarico e della mammella, in contrasto con i corrispondenti tessuti normali in cui l'espressione è sempre assente [3], [4].

Funzioni diverse del gene *cripto* in processi biologici fondamentali hanno suscitato notevole interesse: il suo ruolo chiave nello sviluppo embrionale precoce [5], nel differenziamento delle cellule ES [6], [7] e, non ultimo, nella progressione tumorale [8].

Durante il differenziamento delle cellule ES *cripto* svolge un ruolo chiave nel differenziamento cardiaco. In appropriate condizioni colturali le cellule ES possono essere indotte a formare strutture tridimensionali, chiamate corpi embrioidi (EBs) in grado di dare origine ai tre foglietti embrionali (endoderma, mesoderma ed ectoderma) ed a tutte le cellule caratteristiche dei tre foglietti. *Cripto* svolge un ruolo fondamentale nella specificazione di uno di questi tre foglietti, il mesoderma, ed in particolare è strettamente necessario per la formazione delle cellule cardiache. È stato, infatti, dimostrato che cellule ES *cripto*^{-/-} perdono selettivamente la capacità di formare cardiomiociti contrattili mentre acquisiscono spontaneamente, senza l'aggiunta di induttori specifici, la capacità di differenziare in neuroni. Inoltre, le cellule ES *cripto*^{-/-} differenziano efficientemente in neuroni dopaminergici e sono in grado, impiantate nel cervello di ratti parkinsoniani, di recuperare il fenotipo senza dare origine alla formazione di teratomi [9].

Cripto, quindi, agisce come interruttore molecolare: la sua presenza rende possibile il differenziamento delle cellule ES in cardiomiociti, mentre la sua assenza rende queste cellule capaci di differenziare in neuroni.

Approcci genetici e biochimici, hanno dimostrato che Cripto funziona come co-recettore di Nodal, un ligando della famiglia del TGF-β [10], [6]. Il *signaling* di Nodal dipendente da Cripto è richiesto nello sviluppo embrionale precoce, esso agisce attraverso il complesso recettoriale formato dal recettore dell'Activina di tipo I (ALK4) e dal recettore tipo II (ActRII); tale complesso, una volta attivato, induce la fosforilazione dell'effettore intracellulare Smad2.

Il progetto di ricerca è stato volto, quindi, all'identificazione e caratterizzazione di molecole che agissero da antagonisti dell'interazione Cripto/recettore Alk4, da utilizzare come inibitori del *signaling* endogeno di Cripto, allo scopo di indurre il differenziamento neurale delle ES e allo stesso tempo di inibire la formazione di tessuti di derivazione mesodermica.

La strategia sperimentale eseguita è stata quella di accoppiare la chimica combinatoriale con il differenziamento delle cellule ES.

Le librerie peptidiche fin dal 1990 sono state ampiamente utilizzate per selezionare nuove molecole attive, determinando un'innovazione nella ricerca molecolare e nella scoperta di nuovi farmaci. Il punto di forza nell'uso della chimica combinatoriale è la sintesi di ligandi ad alta affinità per l'analisi e la manipolazione di interazioni biochimiche. Le librerie peptidiche mostrano numerosi vantaggi, tra i quali: la completa automazione della sintesi, la sintesi di un alto numero di molecole in tempi ridotti e l'ottenimento di popolazioni omogenee di peptidi che ne facilitano la caratterizzazione.

Partendo da una libreria peptidica composta da 27.000 molecole differenti, mediante un saggio di tipo ELISA, è stato identificato un peptide capace di inibire efficacemente il legame di Cripto al recettore Alk4, rispetto ai peptidi di controllo che non interferivano il legame Cripto/Alk4.

È stato dimostrato che il Peptide bloccante è in grado di legare specificamente Cripto e non il recettore Alk4. Inoltre, per determinare il contributo della struttura tetramerica all'attività del peptide bloccante, è stata sintetizzata la forma analoga dimerica e la

trimerica ed è stato dimostrato che tali forme non inibiscono (la forma dimerica) o inibiscono con una bassa efficienza (la forma trimerica) l'interazione di Cripto al recettore, dimostrando, quindi, l'importanza della struttura tetramerica per l'attività del peptide. Come discusso precedentemente, le cellule ES *cripto*$^{-/-}$ differenziano spontaneamente in neuroni; questo fenotipo può essere completamente recuperato dall'aggiunta esogena della proteina Cripto ricombinante in una precisa finestra temporale (0-2 giorni del differenziamento). L'aggiunta di Cripto alle cellule ES *cripto*$^{-/-}$ induce la fosforilazione dell'effettore intracellulare Smad2.

Per prima cosa ci siamo chiesti se il Peptide bloccante fosse in grado di bloccare il *signaling* di Cripto e di mimare gli effetti dell'assenza del gene. Abbiamo, quindi, valutato se il Peptide bloccante fosse in grado di inibire la fosforilazione di Smad2 indotta dall'aggiunta della proteina Cripto. Mediante analisi di *Western blot*, effettuata su estratti proteici da corpi embrioidi (EBs), derivati dalle cellule *cripto*$^{-/-}$ e trattati con la proteina Cripto in presenza di quantità crescenti del peptide bloccante o del peptide di controllo, abbiamo dimostrato che soltanto il Peptide bloccante era in grado di inibire completamente la fosforilazione di Smad2, mostrando livelli paragonabili a quelli delle cellule ES *cripto*$^{-/-}$ di controllo.

La disponibilità di una molecola in grado di antagonizzare l'attività di Cripto potrebbe rappresentare un potente strumento per analizzare il ruolo funzionale di tale gene nel differenziamento delle cellule ES.

Sulla base di queste considerazioni e dai precedenti risultati, il Peptide bloccante è stato usato sulle cellule ES *wild type* per determinare la sua capacità di inibire il *signaling* endogeno di Cripto e quindi di bloccare il differenziamento cardiaco e reindirizzarlo verso quello neurale, così come si osserva in assenza del gene. Per valutare la specificità del Peptide bloccante, è stato analizzato il suo effetto sul differenziamento cardiaco, sia mediante un'analisi morfologica che di immunofluorescenza ed RT-PCR.

Mediante analisi morfologica è stata calcolata la percentuale di inibizione del *beating* (presenza di cardiomiociti contrattili) al tredicesimo giorno del differenziamento confrontando cellule ES *wild type* non trattate e ES trattate con i peptidi (sia bloccante che di controllo) a diverse concentrazioni. L'analisi di tale risultato ha mostrato un'inibizione dose-dipendente del differenziamento cardiaco, soltanto nelle cellule trattate con il Peptide bloccante. Tali cellule non erano in grado di differenziare efficientemente in cardiomiociti ma assumevano un fenotipo simile a quello osservato nelle cellule *cripto*$^{-/-}$, caratterizzato dalla presenza di una fitta rete di neuroni. Questa osservazione è stata confermata sia mediante immunofluorescenza utilizzando un marcatore neurale specifico (Tuj1), che analizzando il profilo di espressione di marcatori specifici del differenziamento cardiaco e neurale.

Studi precedenti hanno dimostrato che le cellule ES *cripto*$^{-/-}$ differenziano efficientemente in neuroni ed, in particolare, in neuroni dopaminergici. Infatti, le cellule ES trattate con il peptide bloccante, generano un numero maggiore di neuroni dopaminergici, come dimostra l'incremento di cellule che risultano positive ad un marcatore per il fenotipo dopaminergico (TH) e dagli alti livelli di espressione non solo di TH, ma anche del gene che codifica il trasportatore della dopamina (DAT). Inoltre, Wnt1, coinvolto nella sopravvivenza e nel differenziamento dei neuroni dopaminergici, è overespresso nelle cellule trattate con il Peptide bloccante. Quindi, il Peptide bloccante non solo è in grado di indurre differenziamento neurale in cellule ES *wild type* ma anche di indurre specificamente la formazione di neuroni dopaminegici.

Inoltre, per confermare la specificità del Peptide bloccante sul differenziamento neurale abbiamo analizzato l'espressione di geni associati sia alla pluripotenza delle cellule ES (Oct3/4 e Nanog) che agli altri foglietti embrionali: mesoderma cardiaco (MLC2a) ed endoderma (α-foetoprotein). Sorprendentemente, sia Nanog che Oct3/4, espressi nella blastocisti e nelle cellule staminali pluripotenti, sembrano essere *down-regolati* nelle cellule trattate con il Peptide bloccante. Questo risultato potrebbe evidenziare una riduzione della presenza di cellule indifferenziate e una maggiore capacità delle cellule ES, trattate con il BP, di differenziare in un fenotipo cellulare, che in questo caso è quello neurale. Tale risultato è molto interessante soprattutto in prospettiva di un potenziale uso terapeutico; infatti, la presenza di cellule indifferenziate si associa ad un rischio maggiore nella formazione di teratomi in esperimenti di trapianto cellulare.

Poiché *Cripto* è un gene chiave nel differenziamento cardiaco, abbiamo inoltre usato il marcatore specifico per la miosina cardiaca (MLC2a), che, come atteso, risulta essere espresso ad alti livelli nelle cellule non trattate o trattate con il peptide di controllo mentre mostra un'espressione ridotta in cellule trattate con il Peptide bloccante. Al contrario, non sono state riscontrate variazioni nell'espressione del marcatore per l'endoderma (α-foetoprotein), indicando, quindi, che il trattamento con il peptide identificato è specifico e non altera il differenziamento di questo foglietto embrionale.

La capacità delle cellule ES *cripto*$^{-/-}$ di differenziare in neuroni e l'assenza di geni del differenziamento del mesoderma cardiaco terminale hanno portato ad ipotizzare che l'assenza del gene potesse essere di aiuto nell'ottenimento di popolazioni più omogenee, caratteristica determinante per l'utilizzo di queste cellule in terapia. Infatti, la maggiore preoccupazione legata al trapianto di cellule ES riguarda la formazione di teratomi da cellule indifferenziate presenti nella popolazione trapiantata. A questo proposito, è stato dimostrato che cellule ES *wild type* e *cripto*$^{-/-}$ trapiantate nel cervello di ratti Parkinsoniani riescono ad integrarsi nel tessuto e sono in grado di differenziare in neuroni dopaminergici e di recuperare il fenotipo. Inoltre, l'analisi istologica ha mostrato la presenza di teratomi nel 75% dei ratti iniettati con le cellule *wild type*, mentre l'assenza di teratomi in quelli iniettati con le *cripto*$^{-/-}$ [9].

Avendo dimostrato che il Peptide bloccante è in grado di interferire efficientemente con il *signaling* di Cripto, riuscendo a bloccare il differenziamento cardiaco delle cellule ES *wild type* e a reindirizzarle verso quello neurale , come accade nelle *cripto*$^{-/-}$, è possibile ipotizzare che il trattamento delle cellule ES *wild type* con il Pleptide bloccante determini una riduzione se non l'assenza di teratomi.

2. INTRODUCTION

2.1 Stem cells and regenerative medicine

Repair of damaged tissues and organs is critical for the survival of any individual. In general, this is accomplished through two distinct, but interdependent, processes. First, dead cells must be replaced by newly generated ones, and then newborn cells must differentiate and become organized in complex patterns that, ideally, should restore the original structure of the injured tissue. The repair properties of any tissue or organ are therefore linked primarily to its intrinsic ability of cell replacement. Consequently, repair is usually more efficient in tissues with a high cell turnover and limited need for pattern reconstruction. For example, cutaneous wounds or bone fractures can leave long-lasting scars, but full functional recovery is usually attained within a few weeks. The adult mammalian central nervous system (CNS), on the other hand, has weak capabilities for both endogenous cell replacement and pattern repair (the rewiring of specifically organized long-distance connections), both of which are essential to achieve a significant functional recovery. Cell transplantation might help us to overcome the intrinsic incapability of the nervous tissue to replace lost elements. In addition, grafted cells might produce some beneficials effects by providing host cells with trophic support. However, neural transplantation will yield little benefit if the donor cells fail to integrate functionally into the recipient brain circuitry. The potential clinical impact of cell replacement in neurological disease has already been shown by the transplantation of fetal mesencephalic cells into patients with Parkinson's disease, which led to long-lasting symptomatic improvement in many treated individuals [11]. However, despite these promising results, significant constraints still hamper the use of fetal cells for neural transplantation. Besides the ethical concerns related to the use of material from aborted fetuses, its viability and purity cannot be reliably controlled. In addition, fetal cells are mostly postmitotic and cannot be expanded or stored for longer than few days, so the preparation of donor tissue has to be synchronized with neurosurgery within a very narrow window of time. The recent breakthroughs in stem cell research have opened up new possibilities for cell replacement therapy to treat neurological diseases [11].

Stem cells are defined as clonogenic, self-renewing progenitor cells that can generate one or more specialized cell types. There are two main types of stem cells: embryonic and non-embryonic. Embryonic stem cells (ES) are pluripotent and can differentiate into a wide range of cell types under appropriated culture conditions; whereas, non-embryonic stem cells (non-ES) are multipotent because their potential to differentiate is more limited to cell types of a particular tissue, being responsible for organ regeneration. ES cells are more prevalent than non-ES cells and have a greater potential to spontaneously differentiate than non-ES cells [12].

Non-embryonic stem cells are also known as adult (postnatal) stem cells, since they derive from adult tissues, such as the bone marrow. Primitive stem cells within the bone marrow niche (hematopoietic stem cell, HSC) posses functional versatility broader than expected ,which is termed trans-differentiation or stem cell plasticity [13]. Stem cell plasticity describes the ability of adult stem cells to cross lineage barriers and to adopt the expression profiles and functional phenotypes of cells unique to other tissues [14]. HSC, expressing markers of the hematopoietic lineage (CD45+) and of hematopoietic stem cells (CD34+), are capable of genomic reprogramming upon exposure to a novel environment and give rise to other tissues such as liver, cardiac muscle, or brain [15]. In addition to HSC, bone marrow also contains mesenchymal stem cells (MSCs), which have the capacity to proliferative

extensively and form colonies of fibroblastic cells [16]. Other adult tissue sources include the nose, muscle, liver, skin, brain, and the retina and limbus of the eye [17]. The term "non-emryonic stem cell" is also applied to less mature source of tissue including umbilical cord blood, obtained at birth, placenta and fetal somatic tissues such as pancreas and liver. Fetal tissues also provide stem cells of an embryonic nature; however, these can only be obtained from the gonads in the first trimester of development [12].

Finally, discovery of cancer stem cells in leukemias and solid tumors has added to the complexity of the stem cell field but stimulated great excitement for both stem cell and cancer biologists [18].

2.2 The Embryonic Stem (ES) cells

ES cells derive, both in mouse and human, from the inner cell mass (ICM) of preimplantation embryos at blastocysts stage [19]. ES cells can proliferate indefinitely *in vitro* as undifferentiated cells either in the presence of relatively high concentrations of leukemia inhibitory factor (LIF) or on the top of a layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) [20]. They can give rise to cell lineages of any type of body tissues/organs, when specific stimuli and/or carefully chosen combination of extrinsic and intrinsic signal factors are provided in the culture (**Fig.1**). The latter property of ES cells is called pluripotency. Several transcription factors have been demonstrated to regulate differentiation of ES cells into specific cell types. Ectopic overexpression of such factors stimulates ES cells to differentiate selectively into specific cell types; furthermore, treatment of ES cells with factors such as ascorbic acid, butyric acid and DMSO also improves the differentiation of ES cells to specific mature phenotypes [21], [22].
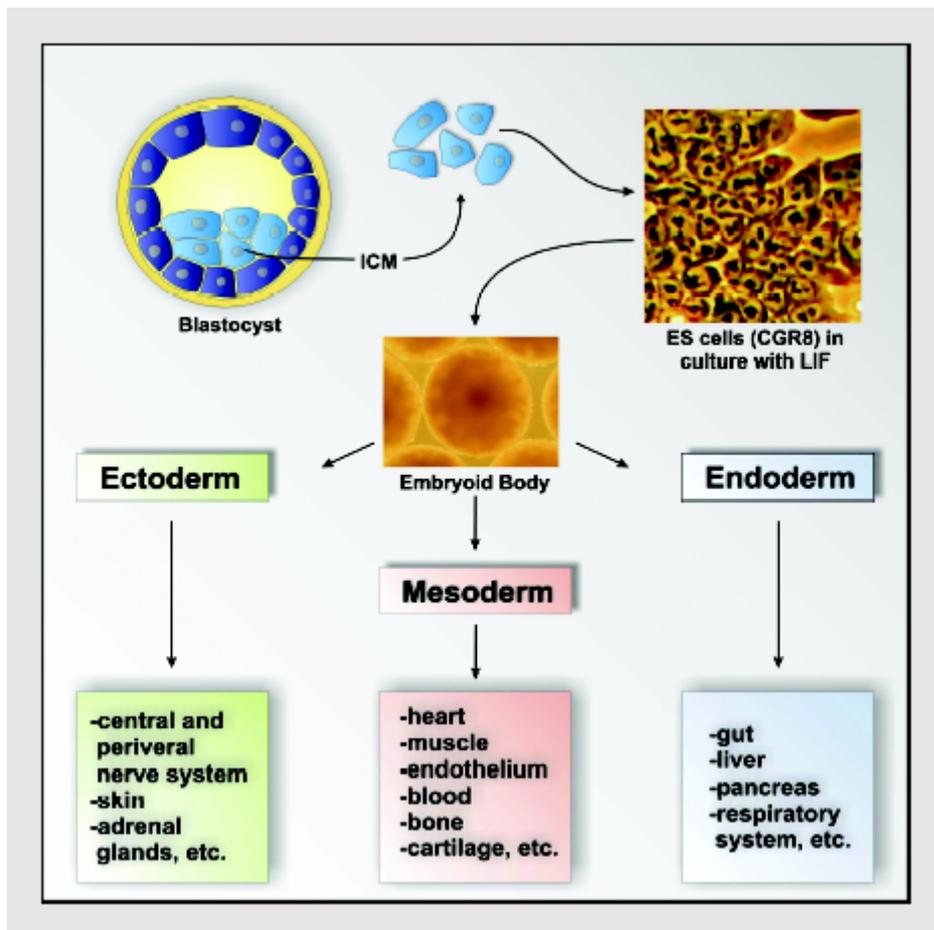
Fig.1: Overview of the derivation of ES cells and their pluripotency. ES cells are derived from the ICM of the early blastocyst and expanded into culture. The Embryoid Body is an intermediate stage during *in vitro* differentiation of ES cells into all three different germ layers and their derivates.

In the absence of LIF, or when removed from feeder layers and transferred into suspension culture, ES cells differentiate spontaneously into multicellular cell aggregates/ embryoid body (ESCAs/EBs), which resemble early post implantation embryos. Formation of EBs promotes spontaneous differentiation of ES cells into the three embryonic germ layers: ectoderm, mesoderm and endoderm, eventually generating both embryonic and adult cell types which include: cardiomyocytes, hematopoietic progenitors, skeletal myocytes, smooth muscle cells, adipocytes, hepatocytes, chondrocytes, endothelial cells, melanocytes, neurons, glia, pancreatic islet cells, primitive endoderm etc. [23].

Worth noting, the routinely used differentiation protocols give rise to a mixture of different cell populations. To harvest a single population of cells, several strategies have been developed such as the use of magnetic bead tagged antibodies or fluorescence labelled antibodies directed against the unique cell surface marker of the desired population. Some protocols employ selective culture conditions that promote the growth of one particular cell type at the expense of the other. ES cells may be genetically engineered to have selection markers or fluorescent markers under the control of lineage/tissue specific promoter, such as a transcription factor that is switched on early during lineage specific differentiation.

2.3 Stem-cell based therapy in Parkinson's disease

 Current research has focused on the use of stem cells to generate neural cell lineages for the treatment of neuronal cell loss associated with neurodegenerative diseases (such as Parkinson or Alzheimer), stroke or CNS injury. Various studies have demostrated the succesful transplantation of either stem cells or precursors into different experimental models of CNS injury and disease, resulting in improvements and/or functional recovery [24], [25], [26], [27], [28].
Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized at a pathological level by a progressive degeneration and loss of: (1) nigrostriatal and mesolimbic dopaminergic neurons, leading to tremor, rigidity and hypokinesia, the classical symptoms of the disease; (2) noradrenergic neurons of the *locus coeruleus*, involved in the progression of the disease; and (3) serotonergic neurons of the raphe obscurus and medial raphe [29]. In addition to neuronal loss, a second prominet feature of PD is the formation of intracellular fibrillar inclusions, called Lewy bodies, which consist of abnormally accumulated proteins, including α-synuclein [30]. The cause of the selective degeneration of specific populations of neurons in PD is unknown, but it has been suggested that increased oxidative stress, mitochondrial sysfunction, and excytotoxic damage are involved in the physiopathology of the disease. Current approaches for the treatment of PD include symptomatic treatment with combined L-DOPA and carbidopa, which increases the synthesis and release of dopamine [31].
Cell replacement strategies in PD have focused initially on the use of fetal mesencephalic tissue for transplantation. The success of this approach in clinical trials has been limited, however, by practical and ethical issue associated with the need for six to seven human fetuses to provide sufficient numbers of dopaminergic neurons for one Parkinson's disease (PD) patient. Using fewer dopaminergic neurons and non-dissociated pieces of mesencephalic tissue after long-term culture and without immunosuppression did not show any therapeutic effect. A high number of dissociated dopaminergic cells are required to achieve therapeutic effects [32]. Grafting of tissues containing diverse types of catecholamine cells, such as chromaphin or carotid bodies, has been proposed as an alternative method for cell replacement. Other approaches have included the engineering of diverse cell types to produce L-DOPA and/or dopamine and the enginnering of a complete midbrain dopaminergic phenotype in multipotent stem/neural progenitors cells [33].
Positive results with intracaudateputamen grafting of human embryonic mesencephalic tissue in PD patients have provided the foundation for the development of novel cell replacement strategies based on the grafting of neural stem cells [29]. Recent data have shown that Embryonic Stem (ES) cells may serve as a source for deriving the optimal dopaminergic (DA) cells to restore the nigrostriatal system [2].
It has been shown that naive ES cells, when grafted at low concentration, develop into normal midbrain-like DA neurons that cause gradual and sustained behavioral restoration of DA-mediated motor asymmetry [2]. Worth noting, previous observations indicated that grafts resulting from high concentrations of ES cells often developed into cells originating from all germ layers giving rise to teratoma formation [34]; thus suggesting that application of low doses of ES cells will result in diluting epidermal or other germ-layer inducing signals and cell-cell contact, resulting in facilitating neural differentiation [2].

The molecular mechanisms as well as the signaling pathways implicated in neural generation in the context of stem cells have not been extensively evaluated and need to be further characterized. Indeed, both the growth and the differentiation potential of ES cells need to be controlled, and the risk associated with the growth of non-neural tissues like teratoma formation needs to be eliminated. Worth noting, many signaling networks that orchestrate the development of ES cells and their differentiation to generate functional hematopoietic, mesenchymal, and epithelial lineages are also implicated in various forms of human cancer. These include signaling pathways activated by the ligand Wnt, epidermal growth factors (EGF), insulin-like growth factors (IGFs), and fibroblast growth factors (FGFs). The pathways activated by each of these ligands regulate lineage establishment and activation of these pathways can contribute to tumorigenesis. Implantation of undifferentiated ES cells leads to formation of benign teratoma in the recipients [20]. This demands a pure population of terminally differentiated cell phenotype. In this respect, new strategies and methodologies need to be developed to isolate the terminally differentiated cells. For example, ES cell implants can be tagged with some kind of death signals in such a way that when they start to form tumors by accident, or when they start to cause severe complications, they will be cleared off from the body leaving the host unaffected [35]. Moreover, ES cell-derived cellular grafts might be rejected due to immunogenicity of the transplanted cells. ES cells can be easily manipulated genetically to overcome the problem of immune rejection so that lifelong pharmacologic immunosuppression will not be needed. However, although the therapeutical cloning approach appears to be promising for avoiding possible immunological problems by the recipients, this approach bears several ethical problems that should be solved by social consensus before clinical studies in this direction can be initiated [35].

Based on these considerations, the introduction of developmental control genes into stem cells may be a useful strategy to direct their differentiation and eventually promote controlled differentiation. Actually, the developmental fate of a cell can be determined by insertion or deletion of genes encoding factors that control various stages of development. Although this approach may represent a powerful strategy, it suffers from several adverse side effects due to clonal variance, promoter dependence and the apparent ability of some ES cells to silence the expression of transgenes [36]. To overcome these problems, a great effort is made at identifying secreted molecules capable of promoting controlled differentiation of stem cells. For example, a good proportion of neural differentiation is achieved by treatments of multi cellular aggregates derived from ES cells (Embryoid Bodies, EBs) with retinoic acid, in the presence of serum [37] or by co-culture of ES cells with a particular stromal cell line, PA6 [27]. Although these methodologies can improve the efficiency of neural cell generation, they both show some limits. The action of retinoic acid is pleiotropic and of indeterminate physiological relevance; it is therefore preferable to avoid RA treatment. On the other hand, the effect of PA6 cells is attributed to an undefined neural inducing activity; the factor(s) responsible have been termed stromal cell-derived inducing activity (SDIA) and the nature of the factors that mediate neurogenesis is still unknown.

Studies during the last decade have established that members of the transforming growth factor-β (TGF-β) superfamily play a critical role in cell morphogenesis and lineage specification in the developing brain [38]. Thus, regulation of neuronal growth, differentiation and specification depends on the activity of these growth and morphogenetic factors, their antagonists, as well as their target membrane-bound

receptors and intracellular signaling proteins [38]. *Smad4* and *Cripto* encode key components of the TGF-β signaling pathway that regulates multiple aspects of embryogenesis, including mesodermal and epidermal cell development [39] and it has been suggested that inhibition of these intrinsic and extrinsic signals can favor neuroectoderm development [38].

Particular attention is focused on *cripto* gene. Indeed, disruption of *cripto* in ES cells results in an enhanced ability of these cells to generate neurons in the absence of any specific inducing factors; furthermore, *in vitro* differentiation of *cripto*^{-/-} ES cells results in increased dopaminergic differentiation and, upon transplantation into Parkinsonian rats, it results in behavioural and anatomical recovery with no tumor formation [9].

2.4 Cripto and the EGF-CFC family

Cripto is the original member of a family of vertebrate signaling molecules, the EGF-CFC family, which includes: human, mouse (*cripto* and *cryptic*), chicken (*cripto*), *Xenopus* (*FRL1*) and *Zebrafish* (*oep*) [40], [41], [42], [43], [44], [45], [46]. These factors have key role in specific developmental processes such as anteroposterior (A-P) axis formation and left-right specification [47], [48], [49], [4], [50]. All EGF-CFC proteins contain a signal sequence for extracellular secretion, a variant Epidermal growth Factor (EGF)-like motif, a novel cystein-rich domain called the CFC domain (for cripto-FRL1-Cryptic) and a C-terminal hydrophobic region. Initially described as secreted molecules, members of this family are extracellular membrane proteins, anchored to the lipid bilayer through a glycosylphosphatidylinositol (GPI) moiety [51] (**Fig.3**).
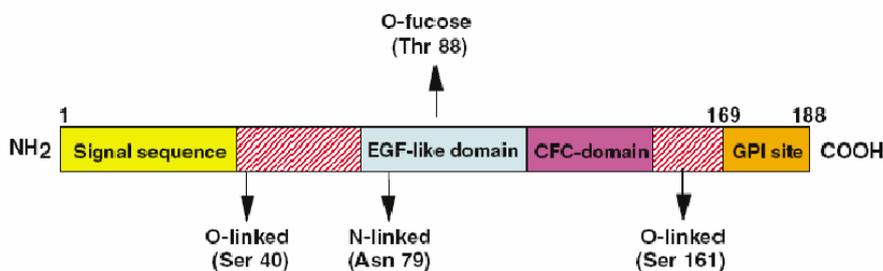
Fig.3: Schematic representation of the domains structure of the EGF-CFC proteins. Yellow: signal peptide; cyan: pseudo-EGF motif; violet: CFC motif; orange: hydrophobic C-terminus. Bottom: consensus sequences of EGF-like, pseudo-EGF, CFC motifs.

Although all the members of the family show a high level of primary sequence identity in two of the central motifs termed EGF-like and CFC motifs, they share a weak overall primary sequence identity. It is worth noting that the sequences at the N- and C-terminal regions of the EGF-CFC proteins are apparently unrelated. Therefore, based on their weak overall primary sequence identity, it has been suggested that Cripto, Cryptic, FRL1 and Oep define four distinct members in the EGF-CFC family [44] (**Fig.4**).
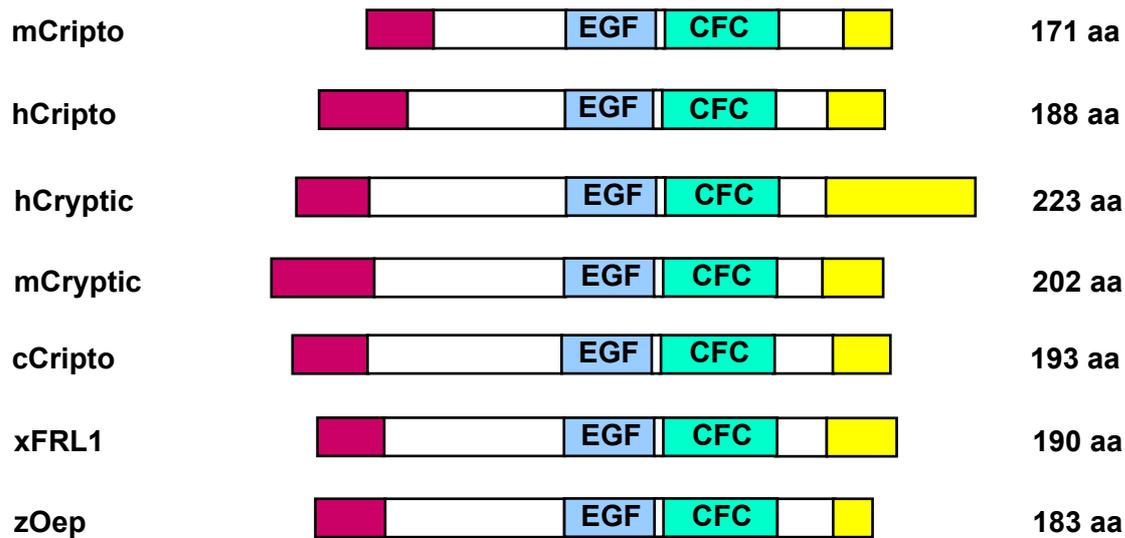
Fig.4: Sequence alignment of the EGF-CFC proteins: mouse Cripto (mCripto), human Cripto (hCripto), human Cryptic (hCryptic), mouse Cryptic (mCryptic), chicken Cripto (cCripto), frog FRL1 (FRL1) and zebrafish Oep (Oep). Blue and green shaded areas indicate the EGF and the CFC domains, respectively.

The *cripto* cDNA encodes a protein of 171 amino-acids with a predicted molecular mass of 18 kDa. However, its apparent molecular weight under denaturing conditions is ~23 kDa, due to post-translational modifications (N- and O-glicosylation). Mouse Cripto is a membrane-bound protein containing two structural consensus required for glycosilphosphatidylinositol (GPI) anchoring to the cell membrane: a C-terminal hydrophobic region and a sequence matching the consensus GPI cleavage and attachment, consisting of a tripeptide with small amino acids in the first and third positions, ~30 amino acids upstream the C-terminus of the protein. Accordingly, it has been shown that Cripto protein can be released from the membrane upon treatment with phosphatidylinositol phospholipase C (PI-PLC) [51]. Only mouse Cripto and *Zebrafish* Oep have been clearly demonstrated to be membrane-anchored, while the subcellular localization of the other members of the EGF-CFC family still remains to be clarified.

2.4.1 Signaling pathways and biochemical activity of Cripto

Cripto was originally described as an EGF-related growth factor [40]. While in the canonical EGF motif, three loops (A, B and C) can be recognized, the variant EGF-like motif in the Cripto protein lacks the A loop, possesses a truncated B loop and has a complete C loop which results in a structure that does not bind to the EGFR family of receptors [52]. However, Cripto does cross-talk with ErbB4 receptor and FGFR-1 indirectly, stimulating the tyrosine phosphorylation of these receptors and contributing to the subsequent stimulation of growth [52], [3].

Molecular genetic experiments in mice, *Xenopus* and *Zebrafish* point to a strong functional link between the EGF-CFC proteins and Transforming Growth Factor beta (TGFβ)-ligand Nodal [47], [4]. In particular, mouse embryos deficient in Nodal activity or *Zebrafish* lacking the Nodal-related genes *cyclop* (*cyc*) and *squint* (*sqt*) exhibit defective gastrulation phenotypes very similar to those of *cripto* or *oep* mutants, respectively, thus suggesting that Cripto and Nodal proteins act in the same pathway [53], [10]. Both receptor reconstitution experiments and coimmunoprecipitation assays have helped to unravel the biochemical interactions of Cripto, Nodal and the Activin typeI serine/threonine kinase receptors (Alk4 or Alk7). Receptor reconstitution experiments in *Xenopus* embryos have shown that Cripto interacts with Alk4 to permit Nodal binding to the Alk4/Activin typeII serine/threonine kinase receptor (Act-RII) complex, leading to Smad phosphorylation [6], [54]. In addition, Cripto was shown to be implicated in Nodal signaling via the orphan receptor Alk7, since its expression enhances the ability of Alk7 and ActRIIB to respond to Nodal ligands [54]. Finally, a purified recombinant Cripto protein has been shown to directly bind Nodal as well as the Alk4 and Alk7 receptors in the absence of any coreceptor, thus suggesting a cooperation mechanism between these two receptors and Cripto in mediating Nodal signaling [55]. Interstingly, site-direct mutagenesis experiments demonstrated that Cripto binds to Alk4 through its CFC domain to facilitate signaling through the Smad pathway [6], [39]; whereas, Cripto binding to Nodal utilizes the EGF motif [6]. Furthermore, recent work indicate that site-specific N-glycosilation and O-fucosylation may be necessary for Cripto function suggesting that post-translational modifications of Cripto could have a role in Nodal signaling regulation [56], [57], [58]. According to the current model, membrane-bound Cripto recruits Nodal to the Activin typeI (Alk4 or Alk7) and Activin typeII receptors complex (ActRII); upon receptor activation, intracellular effectors Smad2 and/or Smad3 are phosphorylated and accumulate together with Smad4 in the nucleus to activate downstream target genes (**Fig.5**).
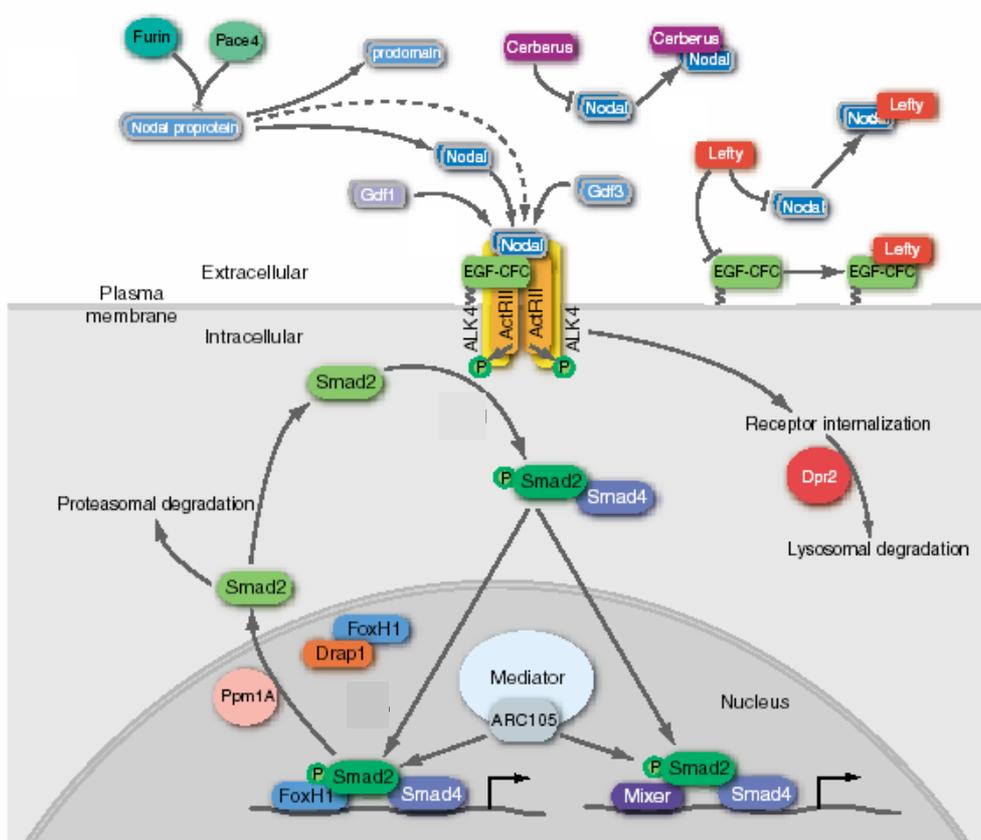
Fig.5: **Schematic representation of Nodal/Cripto/Alk4/Smad signaling pathway.** Cripto acts as a cofactor for Nodal/Alk4 to activate the downstream signaling effectors (Smads).

Worth noting, it has been shown that Cripto can also block activin signaling in cell culture assays and it associates *in vitro* with activin and ActRII (or ActRIIB) to block assembly of an Alk4 complex [59]. It is thus suggested that Cripto could promote tumorigenesis both by inhibiting the tumor suppressor activity of the Activin and by mediating signaling by Nodal or related TGF-β ligands [60].

Finally, Cripto has also been shown to activate non-TGF-β signaling pathways. Indeed, activation of the ras/raf/mitogen-activated protein kinase (MAPK) and PI3K/AKT pathways by Cripto has been demonstrated both in mouse and human mammary epithelial and cervical carcinoma cells; however, the relationships between these pathways and the Nodal pathway is still unclear [61].

2.4.2 Role of Cripto in embryo development and ES cell differentiation

  Early studies of *cripto* were focused on its possible role in cell transformation and tumor progression. *Cripto* expression was first found in human and mouse embryonal carcinoma cells (EC) and male teratocarcinomas and it was demonstrated to be activated in breast, cervical, ovarian, gastric, lung, colon and pancreatic carcinomas, in contrast to normal tissues where *cripto* expression was invariably absent [3]. *Cripto* can function as an oncogene by inducing cell transformation, migration, invasion and

epithelial-to-mesenchimal transition (EMT) in mammary epithelial cells and in breast and cervical carcinomas cells [62]. EMT is one of the key processes in morphogenesis and tumor progression; it involves a dramatic change in cells phenotype by which a well-differentiated and polarized epithelial cell is converted into a mesenchymal cell with a leading edge that facilitates its migration through the extracellular matrix, and thus the colonization of different structures. Interestingly, acquisition of this phenotype is fundamental for the formation of many tissues and organs during embryonic development and constitutes the first step in the metastatic process in tumors of epithelial origin [63].

Besides its role in tumor progression, a great effort has been undertaken to unravel the role of *cripto* in development and ES cell differentiation. In mouse embryos, *cripto* is expressed early in the inner cell mass (ICM) and the throphoblast cells of the blastocyst. At 6.5dpc *cripto* is expressed in the epiblast and the primitive streak stage in the forming mesoderm. Later on, *cripto* expression is associated with the developing heart structures. In 8.5 dpc embryos, *cripto* is expressed in the myocardium of the developing heart tube, whereas, et 9.5 dpc its expression is restricted to the outflow region, conotroncus, of the heart. After 10.5 dpc, no expression of cripto is detected [41]. According to this expression profile, mouse embryos deficient for the *cripto* gene die around day 7.5 of embryogenesis due to defects of mesoderm formation and axial organization [64]. Furthermore, *cripto* mutants exhibit defects in myocardial development as evidenced by the absence of expression of terminal myocardial differentiation genes such as αMHC and MLC2v [5], [65]. These embryos consist mostly of anterior neuroectoderm and lack posterior structures (head without trunk). The letality of *cripto*^{-/-} embryos at early stages, and their gross developmental abnormalities, precludes the investigation of heart morphogenesis in these mutants. To address the role of Cripto in the commitment and differentiation of the cardiac lineage, the pluripotent embryonic stem cells represent a suitable "*in vivo*-like" model to recapitulate early stages of cardiac development. Accordingly, by using embryoid bodies (EBs) derived from *cripto*^{-/-} ES cells, it has been shown that *cripto* is essential for cardiomyocyte induction and differentiation [66]. *Cripto*^{-/-} EBs do not generate contracting cardiomyocytes even during extended culture periods. Wild type (wt) and *cripto*-/- ES cells induced to differentiate by the hanging drops protocol show noticeable differences. *Cripto*^{-/-} derived EBs give off extensive outgrowths of neuritis; in contrast, no wt-derived EBs show neuritic outgrowth. Accordingly, as shown by immunostaining with a pan-neural antibody βIII-tubulin, neurons were detected in most if not all of *cripto*^{-/-} EBs; whereas, βIII-tubulin positive cells were never detected in *cripto*^{-/-} EBs treated with recombinant Cripto protein that, on the contrary, showed extensive areas of MF-20 positive cardiomyocytes (**Fig.7A**).
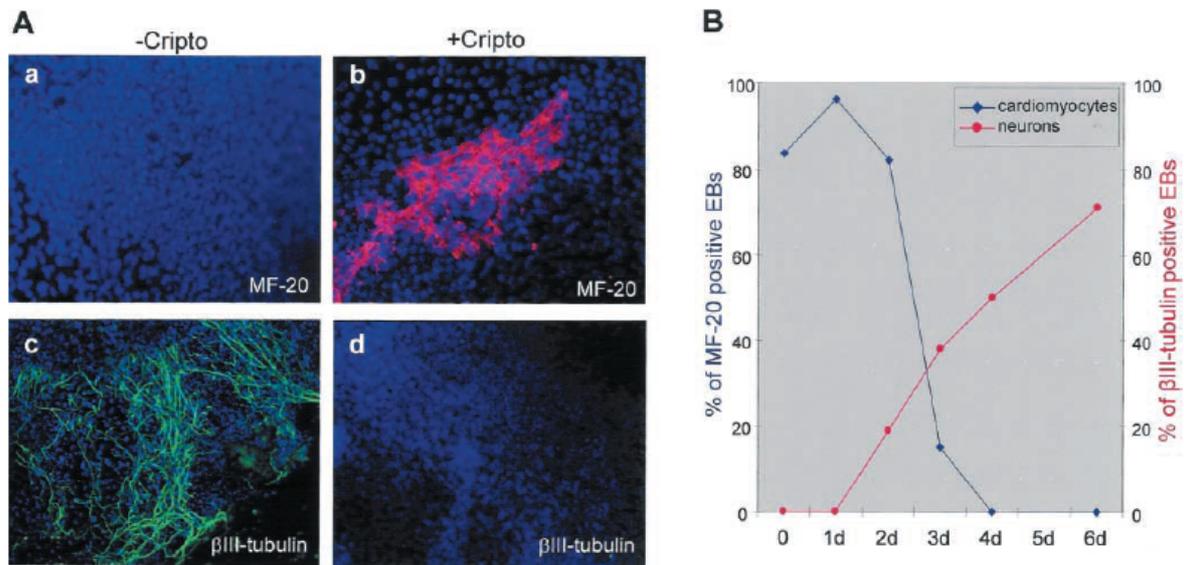
Fig.7: Cripto promotes cardiomyocyte differentiation and inhibits neural differentiation of ES cells according to the timing of exposure. (**A**) Cardiomyocyte versus neuronal differentiation of *cripto⁻/⁻* EBs as revealed by indirect immunofluorescence. 2-d-old *cripto⁻/⁻* EBs, derived from *cripto⁻/⁻* cell line (DE7), were either left untreated (a and c) or treated for 24 h with recombinant Cripto protein (b and d). On day 12 of in vitro differentiation, expression of either sarcomeric myosin or βIII-tubulin was revealed by immunofluorescence using anti-MF20 (red, a andb) or βIII-tubulin (green, c and d) antibodies, respectively. (B) Cardiomyocyte versus neuronal differentiation of *cripto⁻/⁻* EBs-derived cells depends on the timing of exposure to Cripto.

Kinetic experiments indicate that the timing of Cripto signaling required for priming ES cells to cardiomyocyte resembles the competence window of ES cells to acquire a neural fate. Indeed, addition of effective doses of Cripto protein to *cripto⁻/⁻* ES cells during 0-2 day interval restores cardiomyocyte differentiation and, at the same time, results in a dramatic inhibition of neural differentiation (**Fig.8**). Conversely, addition of recombinant Cripto protein at later time points (3-6 day interval) results in progressive impairment of cardiac differentiation, as well as in increased competence of *cripto⁻/⁻* ES cells to acquire a neural phenotype; thus suggesting that different timing of Cripto signaling induces different fates in ES cells (**Fig.8**).
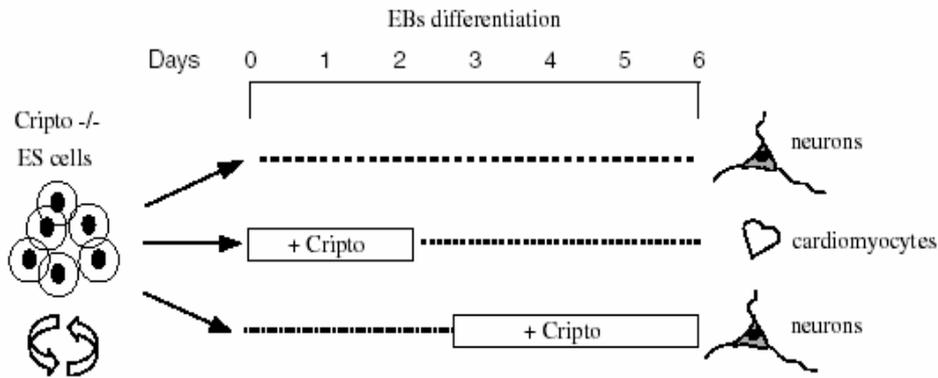
Fig.8: **Different timing of Nodal-dependent Cripto signaling induces different cell fates.** Cardiomyocyte versus neuronal differentiation of *cripto*^{-/-} derived EBs in the presence of serum. Addition of Cripto during the 0-2 days interval of differentiation results in cardiomyocyte induction and differentiation. Addition at later time points (3-6 days) fails to specify the cardiac lineage and results in neuronal differentiation.

Stimulation of *cripto*^{-/-} ES cells with recombinant Cripto has been shown to activate the intracellular effector Smad2. Temporal and spatial regulation of the Smad pathway may be important for normal cardiac development from initial cardiomyocyte differentiation to terminal cardiac morphogenesis. Intracellular activation of Smad2, upon stimulation with recombinant Cripto, require assembly of an active Activin typeI and typeII receptor complex. According to a functional role of Alk4 receptor in Cripto-dependent cardiogenesis, overexpression of an activated form of Alk4 restores the ability of *cripto*^{-/-} ES cells to differentiate into cardiomyocytes [7].

Thus, Cripto acts via Alk4/Nodal signalling pathway to promote cardiomyogenesis and impair neurogenesis in ES cells (**Fig.9**).
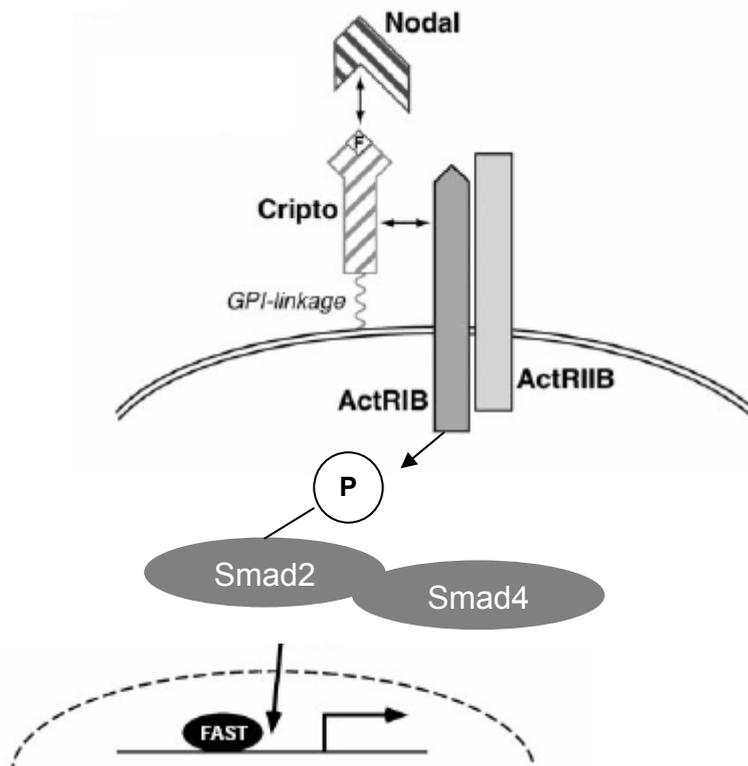
Fig.9: **Schematic representation of Nodal-dependent Cripto signaling in cardiomyocyte differentiation.** Nodal binds directly to Cripto, leading to the assembly of ActRII and Alk4 followed by Alk4 phophorylation and downstream signaling.

2.5 Generation of dopaminegic neurons from cripto$^{-/-}$ ES cells

Recently, Parish C. *et al*. have shown that the absence of *cripto* promotes ES cell differentiation into tyrosine hydroxylase (TH) [9]. Furthermore, treatment of *cripto*$^{-/-}$ derived EBs with Shh and/or FGF8, two factors important for ventral midbrain dopaminergic (DA) neurons, strongly increases the proportion of TH-positive cells as well as the expression of dopaminergic transporter (DAT), compared to wild-type ES cells. Finally, both Wnt1 and Ret, required for survival and differentiation of DA neurons, are over-expressed in *cripto*$^{-/-}$ compared to wild-type EBs. Suppression of *cripto* thus allows ES cells to spontaneously differentiate into neurons, and can increase the cellular pool for dopaminergic differentiation. The increased neural induction in *cripto*$^{-/-}$ ES cells as well as the absence of at least some aspects of terminal mesoderm differentiation, have led to hypothesize that suppression of Cripto may reduce unwanted cell development, thus improving the therapeutic use of ES cells. Accordingly, *cripto*$^{-/-}$ ES cells, when grafted at low density, generate TH-positive cells able to restore behavior in an animal model of PD and, furthermore, do not lead to the formation of teratomas [9]. It is worth noting that transplantation of higher doses of *cripto*$^{-/-}$ ES cells results in generation of terminally differentiated

mesodermal cell types, suggesting that loss of Cripto activity could be compensated in transplant condition by spatial constrain and by the host mouse adult striatum [67]. Cripto is thus a key regulator of ES cell fate; indeed, it is strictly required in an early acting window to negatively regulate neural differentiation and, at the same time, to permit differentiation of ES cells to cardiac fate. Cripto may thus represent an attractive target for future therapeutic approach.

2.6 Aims of the study

  For the success of cell-based transplantation therapies in neurodegenerative diseases such as Parkinson Disease (PD) the control of stem cells differentiation is an important goal. In this regard, Embryonic Stem (ES) cells may be an optimal source for deriving the dopaminergic (DA) cells to restore the nigrostriatal system. Recent findings indicate that the EGF-CFC protein Cripto is a key player in the signaling pathways that control neural differentiation of ES cells; indeed, disruption of cripto in ES cells results in increased dopaminergic differentiation and a reduction in tumor formation upon transplantation in Parkinsonian rats.

Based on these observations, we can hypothesize that inhibition of Cripto activity by affecting Cripto signaling will result in the commitment of ES cells to a neural fate; thus improving the efficiency of generation of neurons from mammalian ES cells. The identification and characterization of such molecules will represent a challenging approach towards the understanding of the molecular mechanisms underlying neural induction in the contest of murine ES cells.

The specific aims of this study are: a) to identify molecules able to inhibit Cripto/receptor interaction by exploiting a powerful experimental approach based on the use of combinatorial chemistry; b) to improve the therapeutic potential of ES cells for cell replacement therapy in PD.

3. RESULTS

3.1 Identification of a novel peptide that antagonizes Cripto/Alk4 receptor interaction

In order to identify new peptides able to inhibit the interaction between Cripto and its receptor Alk4, a combinatorial tetrameric tripeptide library, built with non-natural aminoacids (**Fig.10**), was previously screened.
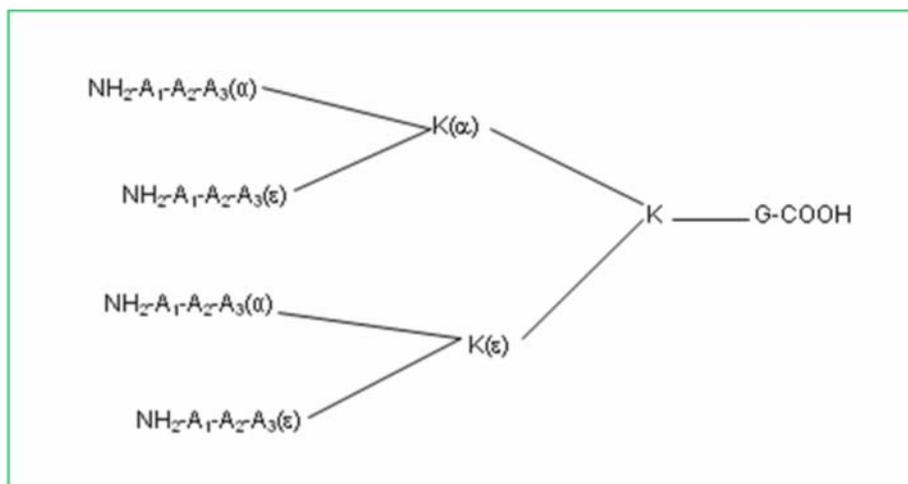
Fig.10: Schematic structure of tetrameric tripeptide library. A letter indicates the unknown positions.

The screening was carried out by an ELISA competition assay whereby the displacement of recombinant Cripto from coated Alk4-receptor by the peptides was evaluated. The screening procedure led to the selection of a peptide that was able to block the interaction of Cripto and Alk4 with IC_{50} value of 250 nM. The Blocking peptide showed up to 80% of inhibition when used at a molar excess of 1.000 with respect to Cripto concentration ($1.8*10-10M$); whereas, an unrelated tetrameric control peptide and a dimeric control peptide did not affect Cripto/Alk4 interaction (**Fig.11**). Moreover, to determine the contribution of the tetrameric structure on peptide activity, the dimeric and trimeric analogues were synthesized. All the peptides were tested at a concentration ranging between 0.1 and 3.5 μM by ELISA-competition assay. As shown in figure 12 the tetrameric structure showed the optimal activity; interestingly, the loss of a single tripeptide strand in the trimer caused a considerable decrease of the activity; whereas, the dimeric peptide did not show any antagonist activity (**Fig.12**).

In order to evaluate whether the identified peptide bound Cripto or Alk4 receptor, both the Blocking peptide (BP) and the control peptide (CP) were coated on microtiter plates and the binding of both Cripto and chimeric Fc/Alk4-receptor to the peptides was assed by ELISA assay. As shown in Fig.13, the BP did not bind Alk4, but it was able to specifically bind Cripto (**Fig.13**).
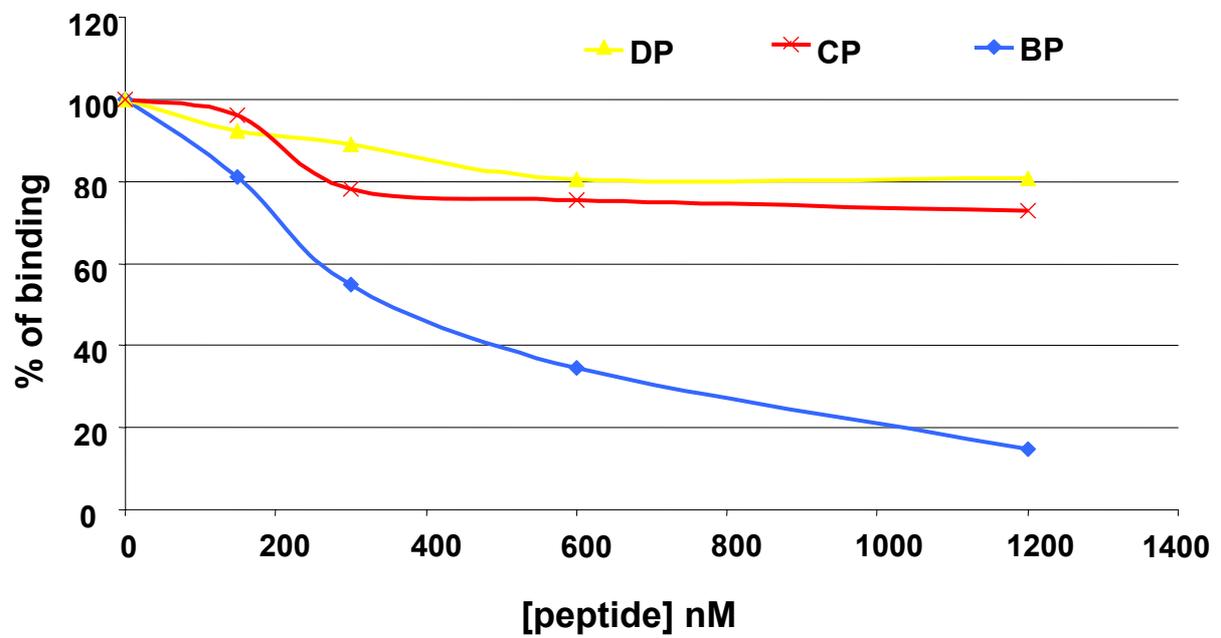
Fig.11: **Dose-dependent inhibition of Cripto with Alk4 by BP in competitive ELISA-based assay.** Cripto Blocking peptide (BP), dimeric (DP) and tetrameric (CP) control peptides were assayed at concentration ranging between 100 and 1200 nM. The results represent the average of three independent experiments.
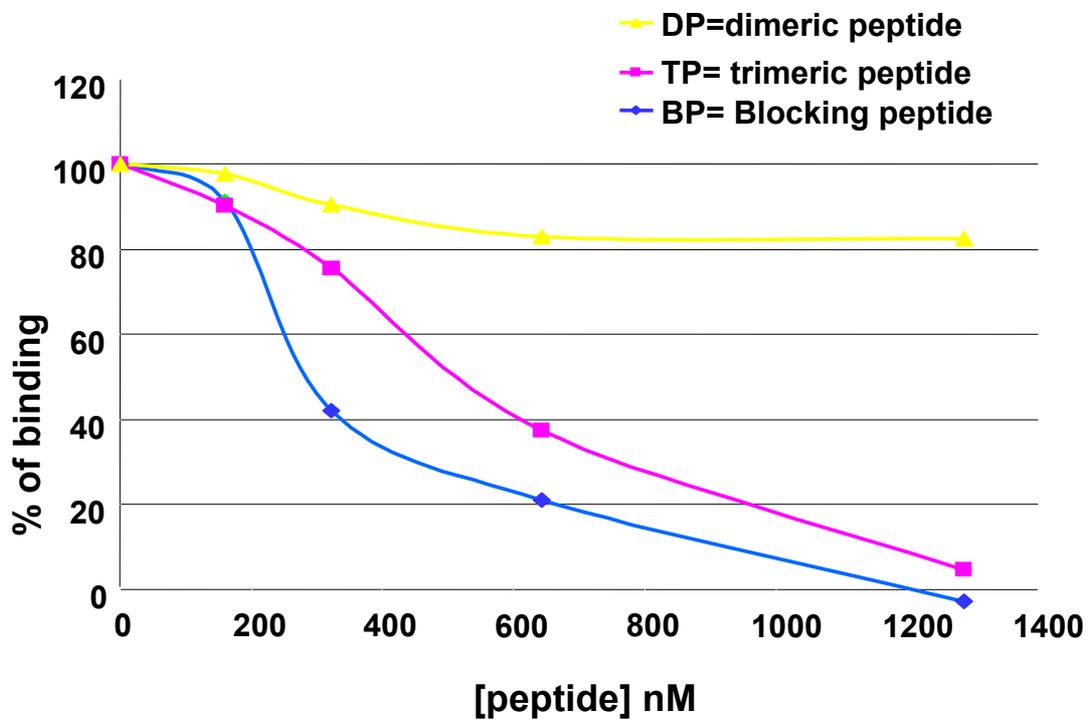
Fig.12: **Influence of sequences and tetrameric structure on peptide activity.** The histograms report the results of ELISA-competition assays on Alk4 performed with the BP and a dimeric (DP) and trimeric (TP) correlated structures.
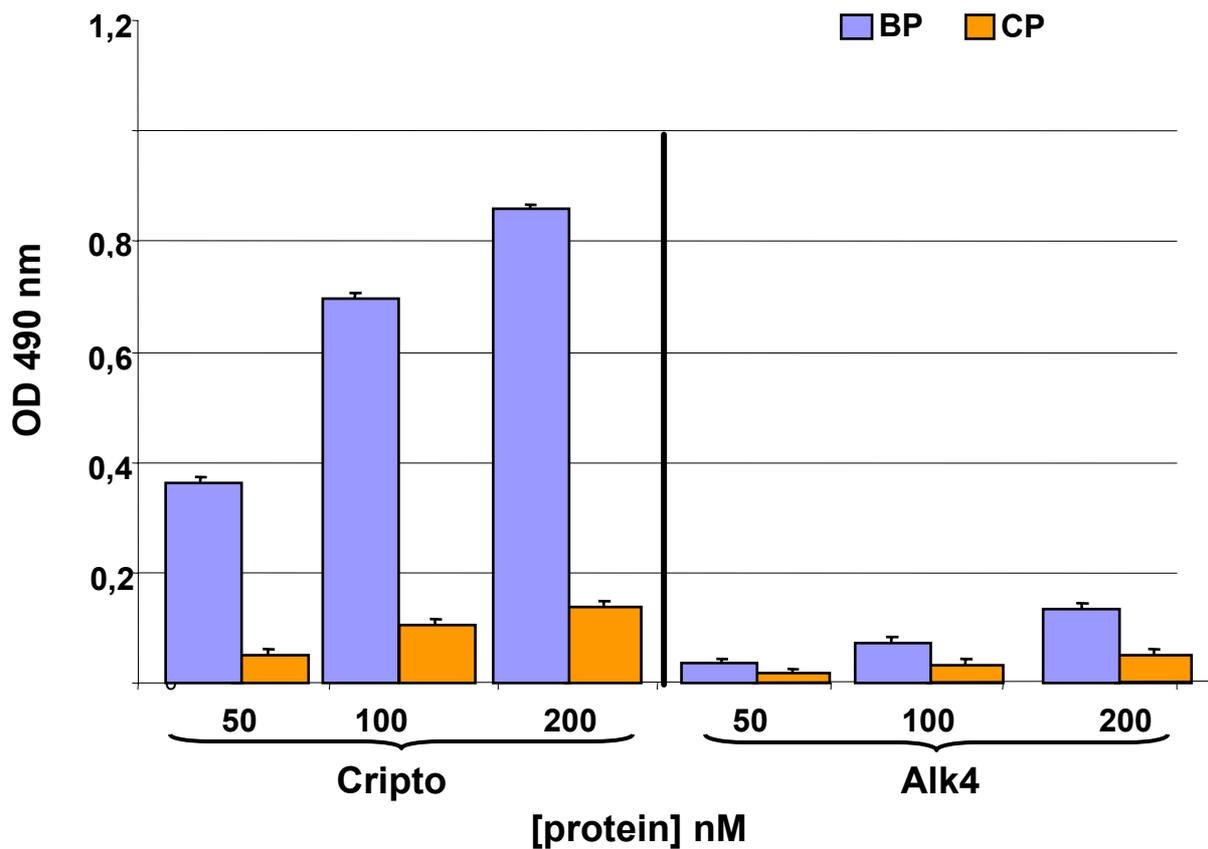
Fig.13: The Blocking peptide (BP) binds Cripto whereas it fails to bind the ALK4 receptor in the ELISA-based assay. Binding assay of BP and CP to Cripto and Alk4 receptor.

3.2 The Cripto/Alk4 blocking peptide inhibits Cripto signaling in ES cells

  The identification of a peptide able to antagonize Cripto/Alk4 interaction may represent a powerful tool to control ES cell differentiation without genetic manipulation.

We first asked if the BP identified was able to block Alk4-dependent Cripto signaling in ES cells and eventually mimic the effect of the absence of *cripto*.

Indeed, previous data from our laboratory showed that Cripto was able to induce Smad2 phosphorylation in an ES cell-based assay, in which the effect of Cripto activity was studied by adding recombinant Cripto protein to *cripto*$^{-/-}$ ES cells at different doses. The data indicated that stimulation *in trans* with soluble Cripto at 0.5μg/mL was sufficient to induce Smad2 phophorilation in 2-day old *cripto*$^{-/-}$ derived-EBs. To directly address the above issue, we evaluated whether the Blocking Peptide was able to block Cripto-induced Smad2 phosphorylation. To this end, 2-day old *cripto*$^{-/-}$ derived-EBs were serum-starved for 3 h and then treated for 20' min with recombinant soluble Cripto protein either alone or in the presence of increasing amount of either Blocking Peptide or control peptides (2.5 μM and 25 μM).

Both the dimeric peptide (DP) and the tetrameric peptide (CP), unable to antagonize Cripto/Alk4 interaction, were used as controls. The western blot analysis showed that, both control peptides did not have any significant effect on Smad2 phophorylation induced by Cripto (**Fig.14**, Blue and green bars). On the contrary, when stimulation with Cripto occurred in the presence of the Blocking peptide, a dose-dependent inhibition of Smad2 phosphorylation was observed. Worth noting, when used at 25 μM, the Blocking peptide completely blocked Cripto signaling (**Fig.14**, Pink bars) and P-Smad2 levels were comparable to the untreated *cripto*$^{-/-}$ derived EBs (**Fig.14**, black bar). These data demostrate that the Blocking peptide was able to specifically inhibit Cripto-induced Smad-2 phosphorilation and open the way to evaluate its effect on ES cell differentiation.
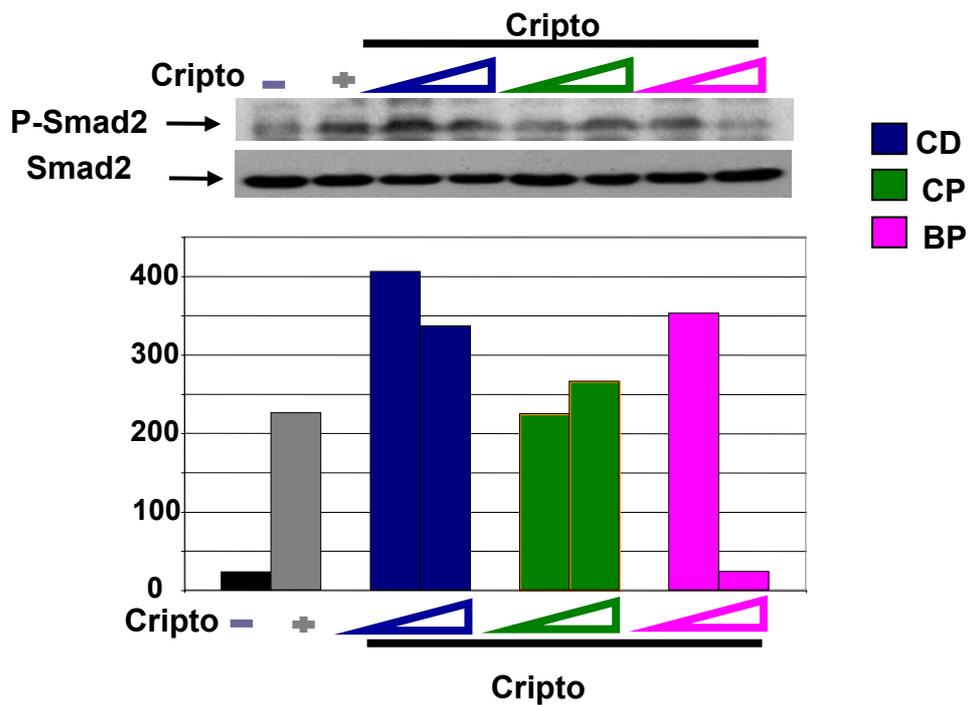
Fig14.: Inhibition of Cripto-induced Smad2 phosphorilation on Cripto-/- ES cells
The selected peptides, in combination with recombinant mouse Cripto, were used in the *cripto^-/-* ES cell differentiation to test their ability to inhibit Cripto-induced Smad2 phosphorilation. The peptides and recombinant mouse Cripto were added directly to 2 day-old ES cell-derived EBs for 20 minutes. Dimeric (DP) and tetrameric (CP) forms were used as negative controls .

3.3 Cripto Blocking peptide is able to impair cardiomyogenesis and to redirect the neural fate of ES cells

The availability of molecule(s) that antagonize Cripto activity may represent a powerful tool to dissect the functional role of Cripto in ES cells differentiation, thus allowing to unravel the functional role of Cripto in the commitment of ES cells to neural fate. Based on these considerations and previous results, the Blocking peptide was used on wild type ES cells to test its ability to inhibit endogenous Cripto signaling and thus impair cardiomyogenesis and induce neurogenesis in ES cells.

We have previuosly identified the precise time window of Cripto signaling which is required to achieve proper cardiomyogenesis. Indeed, Cripto signaling is required in an early and narrow window (0-4 day) to promote cardiomyogenesis.

We thus evaluated the effects of Cripto BP on cardiomyogenesis by adding both the BP and the CP at different time during wild type ES cell differentiation. The biological effect of the peptides was first evaluated by morphological analysis and the numbers of EBs containing beating ares was scored from day 8 to 12 of differentiation.

We then went on to define both the optimal concentrations of the peptides and timing of addition. First of all, since the peptides were dissolved in DMSO, it was mandatory to determine the optimal DMSO concentration to be used. It is well known that DMSO at 1% is a potent inducer of cardiac differentiation of ES cells. For this reason, we have used 0.5% DMSO that, in our experimental conditions, was unable to increase cardiac differentiation. Addition of increasing amount (5 μM to 25 μM) of either BP or CPs to wild type EBs at 0-d, 0-2-d, 0-4-d of differentiation resulted (Blue arrows, **fig.15**) in severe defects of adesion and impaired differentiation. Worth noting, addition of peptides to EBs at 4-d of differentiation (Red arrow, **fig.15**) did not effect cardiomyogenesis. On the contrary, addition of peptides to either 2-d or 2-4-d of differentiation did not affect EBs adhesion and specifically impaired cardiomyogenesis (Green arrows, **fig.15**).
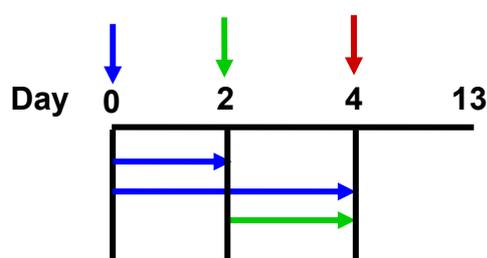
Fig.15: **Schematic representation of peptide addition**. Wild type ES cells were treated with the peptides (BP and CPs) for the indicated periods of time. Blue arrows: peptides addition at: 0-d , 0-2 and 0-4 day of differentiation. Green arrows: peptides addition at 2-d and 2-4 day of differentiation. Red arrow: peptides addition at 4-d of differentiation.

Interestingly, a dose-dependent inhibition of cardiomyogenesis was observed in 13-day old EBs treated, with Blocking peptide (BP), at day 2 of differentiation, as shown by progressive decrease of wt EBs showing rhytmically contracting areas (**Fig.16**). On the contrary, the tetrameric control peptide did not significantly affect cardiomyogenesis, even if used at the highest concentrations (12,5 µM and 25 µM) (**Fig.16**).

DMSO 0.5%	-	+	+	+	+	+	+	+
CP	-	-	+	+	-	-	-	-
BP	-	-	-	-	+	+	+	+
			12.5 µM	25 µM	5 µM	6.25 µM	12.5 µM	25 µM
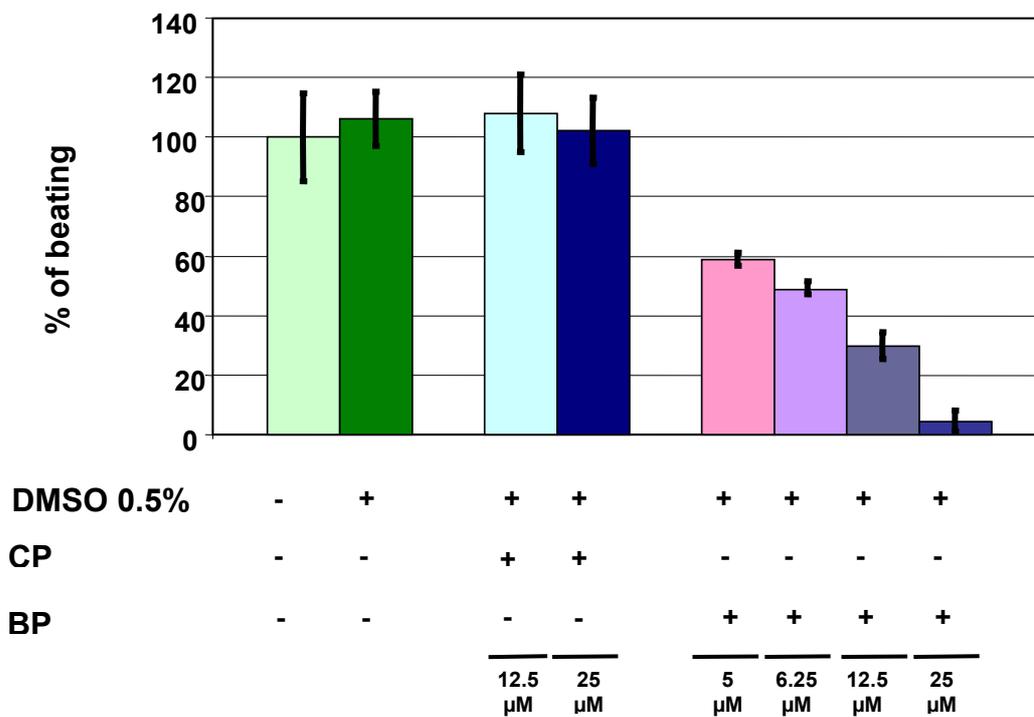
Fig.16: **Effect of BP on cardiomyocytes formation.** Wild type ES cells were treated or untreated both with BP or CP as indicated, and the percentage of beating EBs was scored. The peptide were added raging 5 µM and 25 µM.

To rule out the possibility of a more general effect of the Blocking peptide on ES cell differentiation and/or viability, the effect of peptides was verified on neuronal differentiation of ES cells. Indeed, as previously discussed, in the absence of Cripto ES cells spontaneously differentiate into neurons and the competence window of ES cells to acquire a neural fate correlates with the timing of Cripto signaling required for priming cardiomyogenesis [7]. For this reason, it was hypothesised that the Blocking peptide, if specific and effective, could re-direct ES cell fate and promote neurogenesis. To evaluate whether the BP was able to promote neural differentiation in ES, cells 2-day old wild type EBs were treated with 25 µM of either Blocking peptide or tetrameric control peptide. Interestingly, when wild type EBs treated with Blocking peptide, were plated onto an adhesive substrate, a population of cells with a neuron-like morphology was observed at day 13 of differentiation. This characteristic

morphology was never observed either in wild type EBs or in wild type EBs treated with control peptide. To confirm that those cells were indeed neurons, immunofluorescence analysis (IF) was performed by using antibodies that recognize the neuron-specific form of class βIII tubulin (Tuj1). To better define the activity of the Blocking peptide we have arbitrarily chosen four grades of neuronal differentiation on the basis of areas of Tuj1-**ir** (**i**mmuno**r**eactive) cells (**Fig.17A**), ranging from the absence of neuronal differentiation (Grade 0) to full neuronal differentiation (Grade 3) where an extensive ares of Tuj1-ir cells surrounded the EBs. The presence of either few isolated neurons or small areas of Tuj1-ir cells defined intermediate phenotypes, Grade 1 and Grade 2, respectively.

Immunofluorescence analysis, obtained from three independent experiments, clearly indicated that treatment of ES cells with Blocking peptide induced extensive neuronal differentiation, showing Grade 3 phenotype in about 50% of the EBs scored. On the contrary, EBs treated with control peptides failed to efficiently differentiate into neurons, as shown by the presence of small, if any, areas of Tuj1-ir cells (**Fig.17B**); accordingly, most of the EBs showed a Grade 0 phenotype, thus confirming the specific effect of the Blocking peptide in promoting neuronal differentiation.

To support the morphological as well as the IF analysis, we have evaluated the expression profile of both neuronal and cardiac markers by semiquantitative RT-PCR, on RNAs extracted from 13 days-old EBs treated with either control, Blocking peptide, or treated with DMSO alone.

Having shown that *cripto* deficiency promotes the differentiation of neurons but impairs the cardiomyocyte differentiation, we questioned if the Blocking peptide was able to redirect specifically the differentiation to neural fate and what kinds of neuron subtypes were generated. We first analyzed the expression of the pan-neural NFM marker which clearly increased in EBs treated with the Blocking peptide.

Furthermore, the expression of both the tyrosine hydroxylase (TH) which is a gene relevant to the catecholaminergic and especially to the midbrain dopaminergic (DA) neuronal phenotype as well as of DA transporter (DAT), which is turned on late in DA cell differentiation, were significantly increased upon treatment with BP.

RT-PCR analysis also revealed that Wnt1 mRNA, required for survival and differentiation of DA neurons, is over-expressed in EBs treated with Blocking peptide. All together these data indicate that the Blocking peptide not only incresed the capacity to generate neurons but can also generate increased numbers of DA neurons; furthermore, increased Wnt1 expression levels, may result in proliferation and terminal differentiation of DA neurons.

Furthermore, to asses the specificity of the Blocking peptide on neural differentiation, we also analyzed the expression of genes associated both with pluripotency of ES cells and the two others germ layers (mesoderm and endoderm) (**Fig.18**). To this end, we evaluated the expression profile of Oct3/4, Nanog, MLC2a and α-foetoprotein. Oct3/4, a member of the POU family of transcription factors, and Nanog, an NK-2 class homeobox transcription factor, are expressed in the inner cell mass of blastocyst and in pluripotent stem cells, and they are down-regulated upon ES cell differentiation [68], [69]. In this experiment, both Oct3/4 and Nanog appeared down-regulated in EBs treated with the Blocking peptide.

Since Cripto has a key role during cardiac mesoderm formation, we used a specific marker gene for cardiomyocytes, the myosin light chain 2a (MLC2a). As expected, MLC2a was expressed at higher levels both in untreated EBs and EBs treated with control peptide, and was down-regulated in EBs treated with the Blocking peptide.

On the contrary, expression levels of the endoderm-specific marker α-foetoprotein, were comparable in both BP and CP treated EBs.

The increased neural induction in wild type EBs treated with the Blocking peptide as well as the absence of at least some aspects of terminal cardiac differentiation have led to hypothesize that suppression of Cripto may reduce unwanted cell development, thus improving the therapeutic use of ES cells.

A

Grade 0

Grade 1
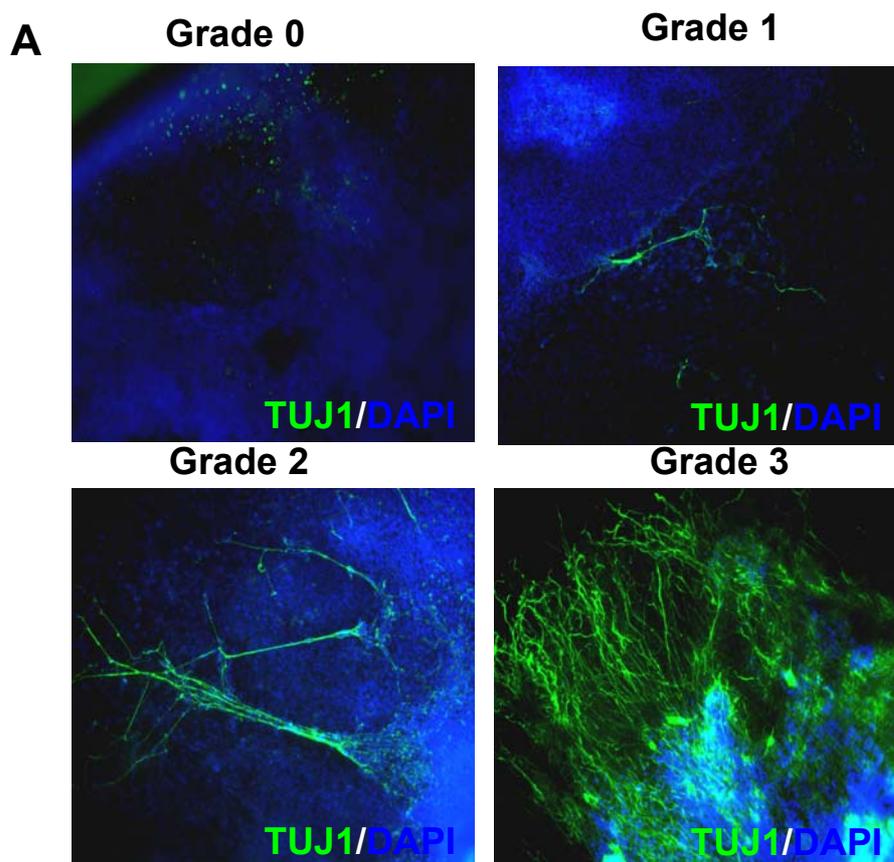
Grade 2

Grade 3

Fig17.: Grades of neural differentiation. 13-day old EBs treated with either control peptide and Blocking peptide , used at 25µM, were analysed by immunofluorescence using antibodies against class III β-tubulin (Tuj1) (**A**) and were calculated the grades of neural differentiation(**B**).

B

DC= dimeric control peptide   CP= control peptide   BP= blocking peptide
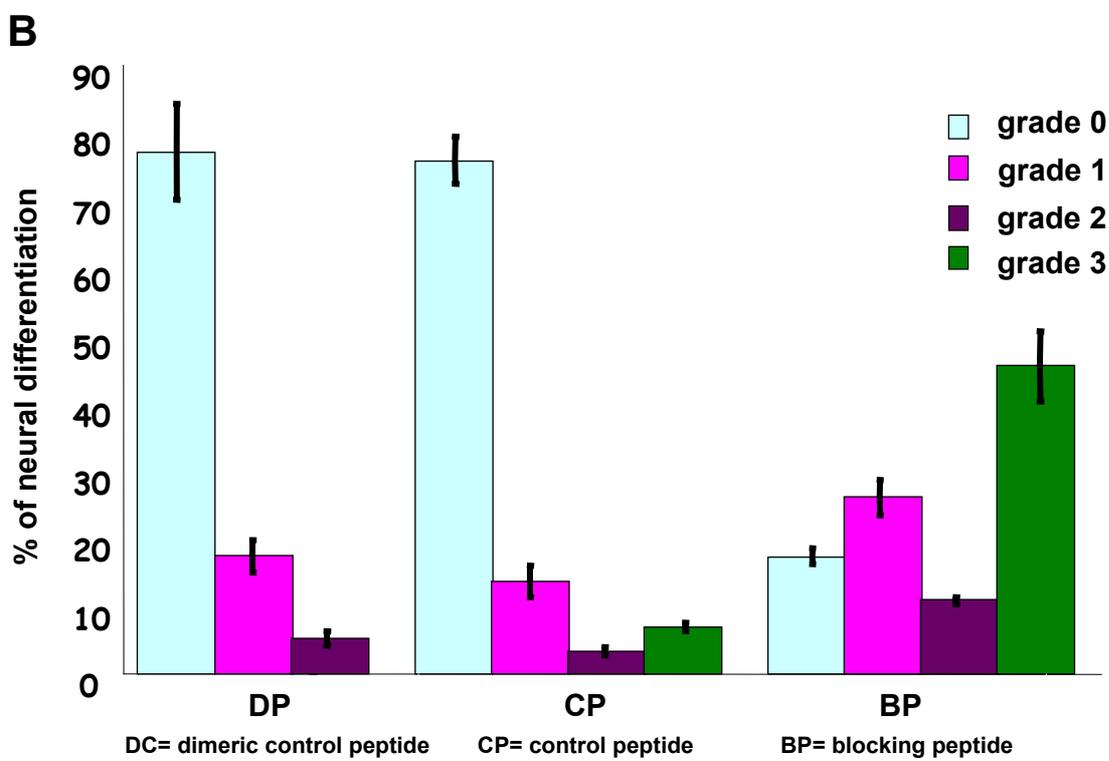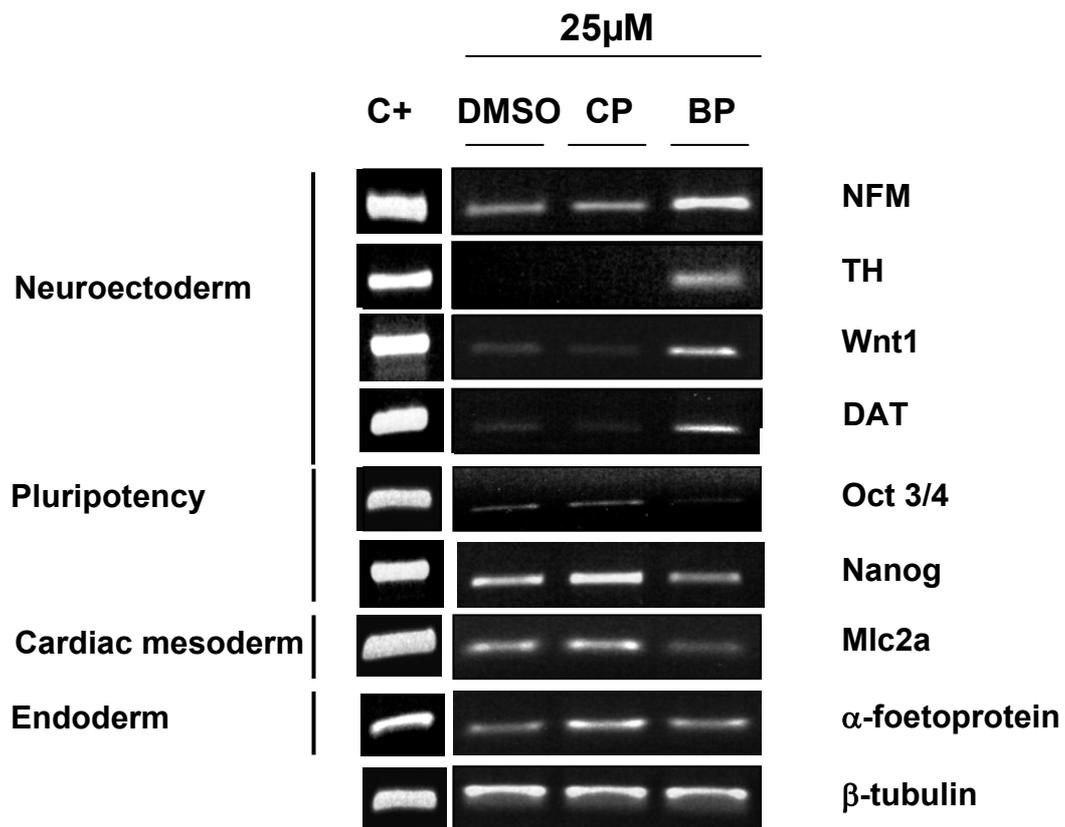
Fig.18: **In vitro differentiation of wt EBs treated with Blocking peptide (BP) increases the number of dopamine cells by increasing neuronal precursors.** Polymerase chain reaction showed: upregulation of NFM, TH, Wnt1 and DAT in EBs treated with BP; downregulation of Oct3/4, Nanog and Mlc2a in EBs treated with BP; no alteration of α-foetoprotein expression. β-tubulin was used as housekeeping gene

3.4 Treatment of ES cells with Blocking peptide increases DA cells

To establish whether blocking Alk4/Smad2 dependent Cripto signaling would enhance the potential of ES cells to generate dopaminergic neurons, the peptides were used in 14day mouse ES cells dopaminegic differentiation protocol [70].
Briefly, with this protocol the cells were plated al low density onto stromal cells (PA6) for a period of 5 days. Cultures are then exposed to Shh and FGF-8 to push cells towards a DA fate for 3 days and then a further 3 days in the presence of Shh, FGF-8 and bFGF to induce differentiation and expand the pool of DA cells. During 1-11 days of differentiation, the Blocking peptide and the control peptides were added every two days at a concentration of 12,5µM. The analysis was performed by immunofluorescence on 14-day old spheres generated by this protocol using both a specific pan-neural marker (Tuj1) and a dopaminergic specific (TH) marker (**Fig.19A**). Following the differentiation protocol treatment of ES cells with Blocking peptide, but not control peptides, increased the percentage of Tuj1 per sphere (**Fig.19B**) and, furthermore, the percentage of TH per sphere (**Fig.19C**).
The data provide, for the first time in our knowledge, direct evidence that a Cripto/Alk4 Blocking peptide is a potent inducer of dopaminergic differentiation.

A

B

%TH+/Tuj+ spheres

C

TH cells/um sphere (%change control)
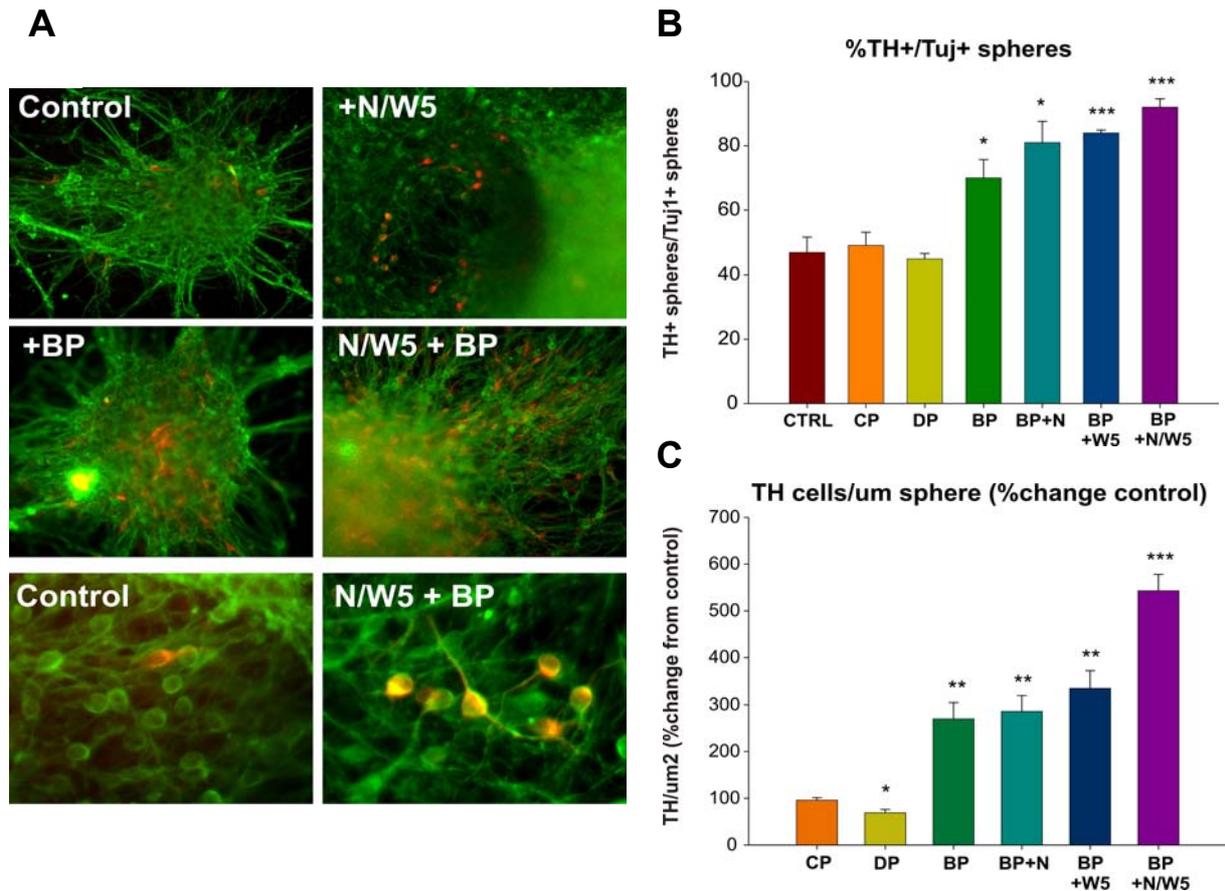
Fig.19: Effects of Cripto Blocking peptide on Dopaminergic Differentiation. Treatment of wild type ES cells with the BP, following the 14 day differentiation protocol, increased the %Tuj+spheres and % of TH/spheres. The actions of the BP on TH+ cells were predominantly a result of incresed neural induction (as reflected in %TH/Tuj). Additionally, the BP incresed the number of TH cells per spehere: 5fold increse in TH cells/sphere under optimal BP conditions).

4. DISCUSSION

In this study we have used the ES cells as a source for obtaining midbrain DA neurons and to eventually improve the use of stem cell-based therapy in rat models of Parkinson's disease. Although the ES cells differentiate into functional DA neurons [2], they also generate cells of the other germ layers, thus eventually giving rise to teratomas. Because teratoma formation is the expected outcome of transplanted wild type ES cells [71], reducing the capacity for both ES cells or neural precursors to differentiate into non-neural tissue is desirable for therapeutic neuronal grafts both to optimize functional effects and to limit cell overgrowth [2]. To overcome this limitation, a great effort is made to get insight into the molecular mechanisms which underlay neural induction in ES cells, an issue which is still debated in the literature.

Based on recent findings from our laboratory, showing that disruption of *cripto* in ES cells redirects the differentiation to a neural fate and especially increases dopaminergic differentiation [9], we verified the hypothesis that neural induction in ES cells can be achieved by inhibiting Alk4-dependent Cripto signaling. Our results show that Cripto signaling inhibition is sufficient to induce development of neural cells and, moreover, that the cell population obtained lack some aspects of terminal cardiac mesoderm differentiation.

4.1 The role of Cripto and the default pathway of neural induction

In several animal models, such as *Xenopus,* zebrafish, chick, and more recently in the mouse, a default mechanism of neural induction has been proposed [38]. This model proposes that neural induction is initiated by the inhibition of BMP signaling in the embryonic ectoderm, through the normal activity of BMP inhibitors such as *noggin*, *chordin*, *follistatin*, and *twisted gastrulation*. In mammals, two lines of evidence are consistent with the model of neural induction using mouse ES as model system: first, transplantation at low density of mouse ES cells into the striatum or under the kidney capsule of adult mice predominantly leads to the formation of neural (mid and hindbrain) cell types [34]; second, *in vitro* differentiation of mouse ES cells on stromal feeders [27] or in limiting dilution assays [72] results in development of neural cell populations. In these experiments, the ES cells rapidly acquire a neural cell fate when differentiated at low cell density and/or when BMP signaling is inhibited by BMP antagonists. However, these experiments were based on the generation of neural precursors form ES cells in the absence of Embryoid Bodies (EBs) formation; therefore, preventing early cell-cell contact and a cellular environment for the germ layer development. During my phD project I have evaluated whether Cripto signaling inhibition is sufficient to induce neural differentiation, even in the presence of germ layer formation and cell-cell contact. Our data indicate that blocking Cripto signaling increases neurogenesis and, furthermore, ES cells do not differentiate into multilineage cell types; thus supporting a default model of neural induction in mammals

4.2 Cripto/Alk4 antagonists redirect the neural fate of ES cells

Based on previous observations that Cripto acts as a molecular switch in ES cell differentiation through Alk4 receptor, we hypothesized that inhibition of Cripto activity by affecting Cripto-Alk4 receptor interaction would result in the commitment of ES cells to a neural fate, thus improving the efficiency of generation of neurons from mammalian ES cells. The identification and characterization of such molecules represent a challenging approach towards the understanding of the molecular mechanisms underlying neural induction in the contest of murine ES cells.

Our aim was to use small molecules, such as peptides, able to inhibit Cripto-Alk4 interaction *in vitro*, using an ELISA-based assay. To this end, we have exploited a novel experimental approach based on the use of combinatorial peptide chemistry combined to ES cell differentiation. Peptide libraries have been largely employed to select new active molecules. Starting from 1990, the selection of ligands from combinatorial libraries has determined a revolution in molecular research and drug discovery [73]. The power of combinatorial selection is the design of high-affinity ligands for the analysis and manipulation of biochemical interaction. Among the wide spectrum of opportunity offered by the combinatorial approaches, the peptide libraries represents one of the most utilized. This is due to the numerous advantages that combinatorial peptide libraries offer: the synthesis is completely automated and with few synthetic steps a high number of molecules are obtained; the products of synthesis are highly homogeneous and easy to characterize [74].

Using recombinant Alk4 receptor and Cripto protein we have set up an in vitro ELISA-based assay that was used to screen a tetrameric tripeptide library composed of 27.000 molecules made of non natural aminoacids. Following library deconvolution a peptide was identified which is capable to efficiently inhibit the binding activity of Cripto to Alk4. This peptide, named Blocking peptide (BP), specifically binds Cripto whereas it fails to bind the Alk4 receptor in the ELISA-based assay. The observed activity was closely dependent on the aminoacidic sequence; in fact, the substitution of a single aminoacid with alanine caused the loss of inhibitory activity, as verified by *Ala-scanning* experiment (data not shown). Moreover, the activity of the BP was also structure-dependent; infact, the tetrameric structure show the optimal inhibition compared with dimeric and trimeric structures.

According to its activity as Cripto/Alk4 receptor antagonist, the BP is able to block Cripto-induced Smad2 phosphorilation in ES cells and thus to interfere with Cripto signaling. Nodal/Cripto-dependent Smad2 activation is required for cardiac mesoderm induction, and once this pathway is inhibited, in early stage of wild type ES cell differentiation, neuroectodermal induction is achieved, consistent with the "default pathway" model [75].

The availability of a molecule that antagonize Cripto activity may represent a powerful tool to dissect the functional role of Cripto in ES cell differentiation, thus allowing to unravel the functional role of Cripto in the commitment of ES cells to a neural fate. Indeed, it conceivable that if specific, enough active and non-toxic, this molecule, by inhibiting the endogenous Cripto activity, will induce neural

differentiation of wild type ES cells and may be used to develop methods to improve the generation of neurons from ES cells.

According to our hypothesis, we have shown that the BP is able to mimic the absence of cripto in ES cells and that treatment of wild type ES cells with the BP results in a phenotype which resembles the cripto$^{-/-}$ phenotype. Infact, addition of BP to wild type ES cells efficiently blocks cardiomyogenesis and redirects the differentiation of ES cells to a neural fate. Interestingly, only the cells treated with the Blocking peptide efficiently differentiate into neurons as shown by the overexpression of the pan-neural marker (NFM), as well as the increased percentage of Tuj1 (neural marker) positive cells. Worth noting, suppression of cripto can also increase the cellular pool for dopaminergic differentiation [9]. Like cripto$^{-/-}$ ES cells, the wild type ES cells, treated with Blocking peptide, are able to differentiate efficiently into dopaminergic neurons and show a great percentage of TH positive cells and a high level of TH mRNA. Also Wnt1, a gene required for survival and differentiation of DA neurons, is over-expressed in EBs treated with Blocking peptide, similar to what observed in cripto$^{-/-}$ derived EBs.

Thus, the BP-treated ES cells not only have increased capacity to generate neurons but can also generate increased numbers of dopaminergic neurons. At the same time, as expected, the cardiac fate determination is impaired, as confirmed by both morphological analysis and RT-PCR, which show the down-regulation of cardiac specific markers. Surprisingly, Oct3/4 and Nanog, both expressed in the inner cell mass of the blastocyst and in pluripotent stem cells, appear down-regulated in EBs treated with Blocking peptide. These results highlight a reduction in the number of undifferentiated cells eventually persisting following the differentiation protocol that we used.

The increased neural induction in cripto$^{-/-}$ ES as well as the absence of at least some aspects of terminal mesoderm differentiation, have led to hypothesize that suppression of Cripto may reduce unwanted cell development, thus improving the therapeutic use of ES cells.

One of the greatest tribulations that exists for ES cell replacement therapy, is the development of teratomas from undifferentiated ES cells present within the grafted population. In this regard, Cripto is known to be overexpressed in several tumors [60], [76], [77]. Grafting studies of ES cells (wild type and cripto$^{-/-}$) into an animal model of Parkinson's disease showed that both wild type and cripto$^{-/-}$ ES cells resulted in behavioral improvements in lesioned rats and integration of DA cells throughout the striatum (graft site). More importantly, these studies demontrated that cripto$^{-/-}$ ES cell grafted animals did not show tumor formations, whilst 75% of brains grafted with wild type ES cells showed teratoma formation [9].

Encouraged by our results, showing that treatment of ES cells with the BP promotes dopaminergic differentiation and reduces the percentage of undifferentiated ES cells, in collaboration with the group directed by Dr. Ernest Arenas at Karolinska Institute, we are currently testing the effects of BP on a rat model of PD. We hypothesize that the treatment of ES cells with the BP, prior to transplantation, would block Cripto signaling and thus reduce the risk of teratoma formation.

5. MATERIAL AND METHODS

5.1 ELISA binding and competition assay

The Alk4 receptor (R&D Systems) was adsorbed to 96 microtiter plates (10 ng/well) and incubated over-night at room temperature(RT). After blocking of aspecific sites with 180 µl/well of 1% BSA solution, 20 ng of Cripto-His protein (purified from conditioned medium of Cripto-His over-expressed HEK293 cells) was added in a volume of 100 µl/well in PBS-EDTA-Tween buffer and the plate was incubated for 1h at 37°C followed by 1h at RT. The plate was then incubated with 600 ng/well of IgG anti-Cripro-His (purified from serum of immunized rabbits) for 1h at 37°C and 1h at RT. A diluition 1:1000 of anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (DAKO), was added to the plate (100 µl/well) and further incubated for 1h at RT. Finally, the plate was developed with o-phenylendiammine peroxidase substrate (Sigma Aldrich) and the absorbance was read at 490 nm on a microtiter rearder (Biorad Benchmark).

The dose dependent activity of the peptides, was measured following the above described protocol at concentrations reported in the Results .

5.2 Propagation and maintenance of ES cells

The ES cell lines RI and *cripto*^{-/-} DE7 were throught the study [65]. Wild type and *cripto*^{-/-} ES cells were maintained in the undifferentiated state by culture on mitomycin C-treated mouse embryonic fibroblast (MEF) feeder layers, according to standard protocols [78]. The culture medium consists of high glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsband, CA) containing 15% fetal bovine serum (Hyclone, Logan, UT), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis), 1 mM sodium pyruvate (GIBCO), 1X non essential amminoacids (GIBCO), 2 mM glutamine (GIBCO), 100U/ml penicillin/streptomycin (GIBCO) and 10^3 U/ml leukemia inhibitory factor (LIF) (Chemicon International, Temecula, CA). ES cells were routinely passaged every 2 days, and the medium was changed on alternated days.

5.3 ES cell in vitro differentiation

For in vitro differentiation to cardiomyocytes, ES cells were cultivated in embryoid bodies essentially as described [79], [80] and following the protocol schematised in Figure 20. Briefly, 400 cells in 20 μl culture medium without LIF were placed on the lids of tissue culture dishes filled with PBS and cultivated in hanging drops for 2 days. After further 3 days of culture in bacteriological Petri dishes in culture medium without LIF, the 5 day-old EBs were plated separately onto gelatin-coated 48-well plates for morphological analysis or onto 100 mm tissue culture plates for RT-PCR and Western blot analysis. Rhythmic beating of the EBs, indicating cardiac muscle

differentiation, was monitored starting to day 7 of differentiation, using phase microscopy (DMIRB, Leica Microsystems, Wetzelar, DE).

Embryonic stem cells
cultivated on feeder layer

Cultivation of ES in
hanging drops

0-2 d

Formation of embryoid
bodies (EBs)

2d

Transfer of EBs and
cultivation in suspension

2-5 d

Plating of embryoid bodies to
48-well tissue culture plates

5-12 d
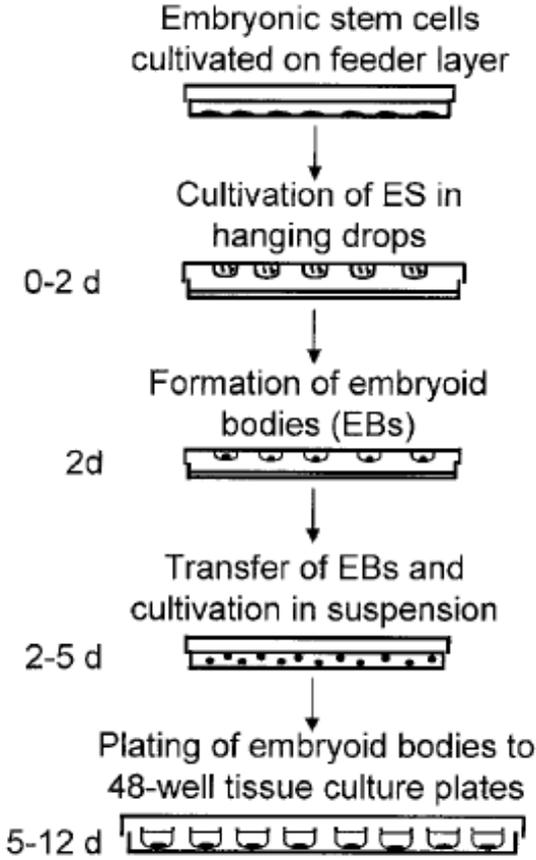
Fig. 20: Schematic representation of the experimental protocol used for ES cell differentiation into cardiomyocytes (adapted from Maltsev et al., 1993).

5.4 Western Blotting

 For cell lysates, the EBs were collected at the indicated time, spin at 900 rpm for 5 min to remove cell debris and lysed in 2x Laemli buffer. Equal amounts of protein, defined by Coomassie staining, were suspended in 2x Laemli buffer and boiled for 5 min. 50µg of cell lysates were resolved on (5-12.5%) SDS-polyacrylamide gel electrophoresis (PAGE). Gels were transferred to a PVDF membrane (Amersham, Buckinghamshire, UK), blocked with 5% milk in Tris-buffered saline tween (TBST) buffer and incubated with primary antibody overnight. After washing three times with TBST buffer, blots were incubated with secondary antibody in TBST-5% milk. The blots were then washed three times with TBST buffer and three times with TBS buffer before color development with ECL substrate colorimetric method (PIERCE), following manufacture instructions.
Rabbit polyclonal anti-Smad2/3, anti-phospho-Smad2 (Ser465/467) (Cell Signaling) were used according to the manufacturer's instructions.

5.5 ES cell in vitro differentiation and peptides treatment

 ES cell differentiation was induced allowing the formation of multicellular three-dimensional EB structures as described in Figure 20. The hanging drop culture assay [80] for cardiomyocyte differentiation was used throughout the study. Two day-old EBs were treated with increasing amounts of both Blocking peptide (5, 6.25, 12.5 and 25 µM) and Control peptide (12.5 and 25µM) and then cultured for the remaining days. The number of EBs containing beating areas was scored at day 13 of differentiation and monitored using phase microscopy (Leica).

5.6 Immunofluorescence

 Adherent 13-day old EBs were fixed for 30' at R.T. in 4% parafolmaldehyde in phosphate-buffered saline (PBS). After fixation, EBs were washed 3 times with 1X PBS for 5' and then incubated with 10% normal goat serum (Dako Cytomation, Glostrup, Denmark)/1% BSA/0.1% Triton X-100 in 1X PBS for 15' at room temperature. The EBs were then washed 3 times in 1X PBS for 5 min and incubated with primary antibody in 10% normal goat serum/1% BSA/1X PBS at the following working dilutions: monoclonal anti-βIII-tubulin (1:400; Sigma-Aldrich). Following primary antibody incubation, EBs were rinsed 3 times in 1X PBS and further incubated with secondary antibodies: goat anti-mouse IgG FITC-conjugated (1:35; DAKO) in 1% BSA/1X PBS for 1h at RT. Finally, EBs were washed 4 times in 1X PBS, counterstained and mounted in VectaShield medium with DAPI (Vector Laboratories). Labeling was visualized by fluorescent illumination using an inverted microscope (DMIRB, Leica Microsystems, Wetzelar, DE); images were acquired on a DC 350 FX camera (Leica).

5.7 RNA preparation and RT-PCR

Total RNA from 13 day-old wild type EBs treated with 25µM of either blocking peptide or control peptide or treated with 0.5% DMSO as control, was extracted using Trizol ultra pure reagent (Invitrogen), according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed with QuantiTect Reverse Transcription Kit (Quiagen). cDNA samples synthesized from 100ng of total RNA were subjected to PCR amplification with appropiate primers.

The PCR was carried out using standard protocols: Taq polymerase (0.1 U/reaction, Euroclone, Pero, Italy), Reaction buffer (1X, Euroclone), $MgCl_2$ (1.5 mM, Euroclone), dNTPs (0.2 mM, Euroclone). Cycling parameters were as follows: denaturation at 94°C for 45 sec, annealing at 56°C for 45 sec and elongation at 72°C for 45 sec. The number of cycles was chosen to select PCR conditions on the linear portion of the reaction curve avoiding "saturation effects" of PCR. Primer sequences, number of cycles and the length of the amplified products are shown in **table1**.

Gene	Cycle number	Primer (sense)	Primer (antisense)	Product size
Oct3/4	26	tcagcttgggctagagaagg	tgacgggaacagagggaaag	250
MLC2a	28	cagacctgaaggagacctattccc	gtcagcgtaaacagttgctctacc	300
α-foetoprotein	30	ccacgttagattcctcccagtgcgt	catacttgttagagagttccgtctc	200
NFM	32	gaaatggaagaaaccctcaca	ccggccttggcctctggttttgg	474
TH	30	tgtcacgtccccaaggttcat	gggcaggccgggtctctaagt	276
DAT	30	gtgggcttcactgtcatcctca	cccaggtcatcaatgccacga	514
Wnt1	30	acctgttgacggattccaag	tcatgaggaagcgtaggtcc	462
β-tubulin	28	gggaggtgataagcgatgaa	cccaggttctagatccacca	150

Table1: Marker genes used for RT-PCR analysis

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ABSTRACTS

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"Recent breakthroughs in stem cell research underscore the importance of controlling stem cell differentiation for the success of cell-based therapies. In this perspective, recent findings from our laboratory indicate that the EGF-CFC protein Cripto is a key player in the signaling pathways that control neural induction in ES cells. Indeed, disruption of *cripto* in ES cells results in an enhanced ability to generate neurons in the absence of any specific inducing factors. We are exploiting a novel experimental approach based on the use of combinatorial chemistry combined to ES cell differentiation, to identify antagonists of Cripto/receptor interaction as new tools to direct neuronal fate specification from mouse ES cells. By using an *in vitro* ELISA-based technology, we have we have screened a tetrameric tripeptide library and identified peptides that are able to inhibit Cripto/ receptor interaction and are currently tested on ES cell differentiation assays. These molecules will be extremely useful to direct ES cell fate specification without the need to manipulate the cells genetically and may thus permit a direct extension of this strategy to human cells."

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induction in ES cells. Indeed, disruption of *cripto* in ES cells results in an enhanced ability to generate neurons in the absence of any specific inducing factors. We are exploiting a novel experimental approach based on the use of combinatorial chemistry combined to ES cell differentiation, to identify antagonists of Cripto/receptor interaction as new tools to direct neuronal fate specification from mouse ES cells. By using an *in vitro* ELISA-based technology, we have we have screened a tetrameric tripeptide library and identified peptides that are able to inhibit Cripto/ receptor interaction and are currently tested on ES cell differentiation assays. These molecules will be extremely useful to direct ES cell fate specification without the need to manipulate the cells genetically and may thus permit a direct extension of this strategy to human cells.

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"During the pathogenesis of cancer, tumor cells revert to an embryonic- like state, where cells divide rapidly and migrate inappropriately through the body. Thus, by studying the interface between normal embryo development and tumor biology, one may gain a better understanding of the molecular events responsible for cancer. In this scenario, the cripto gene, the founder member of the EGF-CFC family has a crucial role. Cripto is expressed both in the Embryonic Stem (ES) cells and during the early phases of embryo development while, in the adult, it is reactivated in a wide range of epithelial cancers. The involvement of Cripto in physiological events associated with development and pathological events linked to tumorigenesis, points out for the search of genes regulated by Cripto. We have studied the expression profile of cripto^{-/-} ES cells stimulated with Cripto protein for 24 hours, by using microarray technology. We are currently characterising the most promising genes by analyzing their expression profile during both ES cell differentiation and mouse embryogenesis.

5. **Lonardo E**., D'Aniello C., Guardiola O., Autiero* M., Liguoro A., Liguori G., Carmeliet* P. and Minchiotti G. **"Cripto signaling in Embryonic Stem cell differentiation: convergent pathways in stem cells and tumorigenesis.**
EMBO/IGB Workshop: "Cell migration, Tissue Invasion and Disease", Capri, 14-17 October 2006.
"In the last years single molecular players of cell migration have been identified and their activity has resulted to be fundamental in the physiological embryonic development and adult life; thus defining an integrated network of molecules and mechanisms that are involved in the control of cell migration both in the embryonic and adult life. In this scenario, the cripto gene, the founder member of the EGF-CFC family has a crucial role. Cripto is expressed both in the Embryonic Stem (ES) cells and during the early phases of embryo development while, in the adult, it is reactivated in a wide range of epithelial cancers, including breast, stomach and colon carcinoma. The involvement of Cripto in physiological events associated with development and pathological events linked to tumorigenesis, points out for the search of genes regulated by Cripto, as a powerful approach to unravel the complex network of molecules

that are active during early embryogenesis and tumorigenesis. To this end, we have studied the expression profile of cripto$^{-/-}$ ES cells stimulated with Cripto protein for 24 hours, by using microarray technology. Indeed, because in vitro differentiation of embryonic cells proceeds as a developmental continuum, this could be considered as a powerful model system to identify key regulators of early embriogenesis. We are currently characterising the most promising genes by analyzing their expression profile during both ES cell differentiation and mouse embryogenesis and eventually by studying their functional interaction with Cripto. "

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"Skeletal muscle has a remarkable regenerative capacity, but any impairment in the restoration of contractile properties after injury can lead to prolonged functional disability. The engineering of skeletal muscle tissue still remains a challenge. Recent findings emerging from different research fields are reinforcing the idea that the same molecules and mechanisms operate during embryonic development and in the adult, under both physiological and pathological conditions.
Cripto is the original member of the EGF-CFC family, which is expressed during early embryogenesis as an important co-receptor for Nodal. Recently, a main effort has been undertaken to identify both the receptors interacting with Cripto and the signaling pathway activated by this interaction. Genetic experiments as well as biochemical approaches, defined a nodal/ALK4/Smad-2 signaling pathway and a nodal- and ALK4-independent signaling pathway that leads to the activation of ras/raf/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K)/AKT signaling pathways implicating the glypican-1 receptor.
Up to know the re-expression of Cripto in adult tissue has been associated with cancer. Our unexpected observation that Cripto was also re-expressed during muscle regeneration prompted us to perform basic molecular studies, both *in vitro* and *in vivo* in mouse muscle injury and diseases models, in order to better understand the precise nature of the biological effects of Cripto, on myogenic cells during post-natal life.
Our preliminary results shown that Cripto induces *in vitro* and *in vivo* cell proliferation, myogenic cell migration leading to *in vivo* faster muscle recovery/regeneration after injury.
Results of the study will be directly beneficial to basic knowledge on cripto activity in striated muscle biology and the identification of a molecular system operative in cripto-induced regeneration will help to understand mechanisms of myofiber regeneration."

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"Recent breakthroughs in stem cell research underscore the importance of controlling stem cell differentiation for the success of cell-based

transplantation therapies in neurodegenerative diseases such as Parkinson Disease (PD). In this regard, Embryonic Stem (ES) cells have been shown to serve as a source for deriving the optimal dopaminergic (DA) cells to restore the nigrostriatal system. However, our knowledge about the molecular mechanisms implicated in the determination of the neural fate in ES cells is still incomplete and thus their differentiation is poorly controlled. Recent findings from our laboratory indicate that the EGF-CFC protein Cripto is a key player in the signaling pathways that control neural differentiation of ES cells; indeed, disruption of cripto in ES cells results in increased dopaminergic differentiation and a reduction in tumor formation upon transplantation in Parkinsonian rats (1).

As an extracellular molecule Cripto is a promising target for therapy (2). We have thus exploited a novel experimental approach based on the use of combinatorial chemistry combined to ES cell differentiation. By using an ELISA-based assay, we have screened a tetrameric tripeptide library composed of 27000 molecules and identified two small molecules that antagonize Cripto/receptor (Alk4) interaction that have been tested on ES cells. Our results show that these blocking peptides, by inhibiting endogenous Cripto activity, improve neuronal differentiation of mouse ES cells and increase dopaminergic differentiation.

These molecules will both provide a useful tool for the understanding of the mechanisms underlying neural induction in ES cells, and hopefully will have important implications for the effective use of stem cells in the treatment of PD, by reducing teratoma formation upon transplantation. Worth noting, the ability to direct ES cell fate solely hrough the use of extracellular factors, without the need to manipulate ES cells genetically, may permit a direct extension of this strategy to human cells.

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A Versatile Method for Differentiation of Multiple Neuronal Subtypes from Mouse Embryonic Stem Cells

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Abstract

Embryonic Stem (ES) cells have recently evoked a great scientific interest, being valuable both for the study of early mammalian development and for regenerative medicine. In this respect, several methods have been recently described to achieve neuronal differentiation of ES cells; however, most of them either require extended *in vitro* culture or give rise to lineage-restricted neuronal differentiation. To overcome these limitations, we have developed an improved method for preparing mouse ES cell aggregates for *in vitro* neurogenesis studies. This protocol relies on the generation of size-controlled Embryoid Bodies (EBs) in a chemically-defined serum free medium without the addition of any specific factors. Neuronal differentiation is assessed by both RT-PCR and immunofluorescence analysis. The expression profile of multiple neuronal-subtype specific markers is analyzed, thus showing that ES-derived EBs undergo a rapid and progressive differentiation toward both glial and neuronal lineages, giving rise to a wide range of neuronal subtypes (i.e. dopaminergic, serotonergic and cholinergic neurons). Indeed, a marked temporal correlation in the onset of gene expression is observed between ES cell differentiation and mouse development. This method provides an efficient platform for studying the molecular mechanisms underlying the events of multiple neuronal subtype specification in ES cells.

Key words: *Embryonic Stem cells, in vitro differentiation, neurons, hanging drops, Embryoid Bodies.*

Introduction

The recent breakthrough in stem cell research have opened up new possibilities for cell replacement therapy; indeed, cell transplantation might help to overcome the intrinsic inability of

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adult tissues, such as the central nervous system (CNS), to replace lost elements. Given their expected capacity to self-renew and differentiate efficiently into the desired cell type, Embryonic Stem (ES) cells represent an invaluable source of different cell types. ES cells are derived from the inner cell mass of pre-implantation embryos; they are amenable to manipulation, enrichment and expansion and retain the developmental potency of embryonic founder cells, being able to differentiate into cells and tissues of all three germ layers both *in vitro* and *in vivo* [1]. Indeed, differentiation of both mouse and human ES cells follows the hierarchical set of signals that regulate embryonic development in the generation of the germ layers and cell types [2-4]; thus, ES cells provide a valid model to understand the complex network of signaling interactions in the mammalian embryo.

In this scenario, understanding how specific ES cell fate decisions are made is crucial not only to fulfill their clinical promise but also to get insights into the molecular mechanisms controlling early events of mammalian development, eventually reactivated in the adult life, in pathological conditions. Systematic manipulation of cell cultures to direct differentiation of ES cells to specific fates requires the development of protocols that maximize control over the extrinsic signaling environment. In this contest, numerous advances were made and several protocols have been described that allow differentiation of ES cells into neurons [5-10]; however, most of them either require extended *in vitro* culture [7] or result in low efficiency of neural induction [8-10]. Worth noting, high levels of neural differentiation are induced either by treatment of ES-derived EBs with retinoic acid (RA) in the presence of serum [5], or by co-culture with a stromal cell line, PA6 [8]. Nevertheless, RA is both a strong teratogen and it is supposed to perturb neural patterning, inducing suppression of forebrain development [11, 12]; moreover, the effect of PA6 cells is attributed to a still undefined neural inducing activity known as stromal cell-derived inducing activity (SDIA) [6].

Recently, efficient neural differentiation of ES cell-derived EBs has been reported to occur in serum-free conditions; however, it mainly results in the generation of telencephalic neurons [13].

Finally, very recently, Conti et al. have described a method to obtain expandable and homogenous neural stem cell population from mouse ES cells that generate lineage restricted neurons [14].

Here we describe a fast and simple method to generate both glial cells and a wide range of neuronal subtypes, such as dopaminergic, serotonergic and motor neurons from mouse ES cells, in the absence of any specific inducing factors or extended cultures.

Material and Methods

Propagation and Maintenance of ES Cells

Mouse ES cell lines R1 [15] and AK7 [16] have been used throughout the study.

Undifferentiated ES cells were grown on mitotically inactivated fibroblast feeder layers and maintained in DMEM (Invitrogen, Carlsband, CA, http://www.invitrogen.com) supplemented with 15% fetal bovine serum (Hyclone, Logan, UT, http://www.hyclone.com), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), 1 mM sodium pyruvate (Invitrogen), 1X non essential aminoacids (Invitrogen), 2 mM glutamine (Invitrogen), 100U/ml penicillin/streptomycin (Invitrogen) and 10^3 U/ml leukemia inhibitory factor (LIF) (Chemicon International, Temecula, CA, http://www.chemicon.com). ES cells were routinely passaged every 2 days, and the medium was changed on alternated days.

Derivation of Neuronal and Glial Cells

High yields of neuronal and glial cells depend essentially on two crucial factors: the generation of size-controlled Embryoid Bodies (EBs) and the use of a chemically-defined medium.

For neural differentiation, nearly confluent 100mm-plate of undifferentiated ES cells were dissociated in a single-cell suspension by using 0.25% trypsin (Invitrogen) in PBS, collected by centrifugation, resuspended in ES medium and plated on 0.1% gelatine coated-100mm dish for 1h

at 37°C to remove most of the feeder cells that quickly attach to the plate. Cells were collected by centrifugation and resuspended at 2×10^4 cells/cm^{-2} in differentiation medium: Knockout Dulbecco's minimal essential medium supplemented with 15% Knockout Serum Replacement (both from Invitrogen), 0.1 mM β-mercaptoethanol, 2 mM glutamine, 100 U/mL penicillin/streptomycin (from now onward KSR medium). EBs differentiation was obtained as previously described [17]. Briefly, 20 µl drops of ES cells (400 cells/drop) in KSR medium were placed on the lid of tissue culture dishes filled with PBS and cultivated in hanging drops for 2 days (Figure 1). The day on which ES cells were induced to aggregate in hanging drops was defined as day 0

of differentiation. Two-day-old EBs were then collected in KSR medium and transferred into 100-mm bacteriological Petri dishes. After further 3 days of culture (Figure 1), 5 day-old EBs were plated in the same medium either onto 0.1% gelatin-coated 100-mm tissue culture plates for RT-PCR or onto 0.1% gelatin-coated 60-mm dish for IF analysis, respectively (Figure 1). At day 10 (5+5) of the *in vitro* differentiation protocol, a population of cells with a neuron-like morphology can be clearly recognized (Figure 2c). Neuritic outgrowths become more complex over the following 3 days (Figure 2d). Finally, 13 day-old EBs were processed for immunofluorescence and RT-PCR analysis (Figure 1), unless stated otherwise.
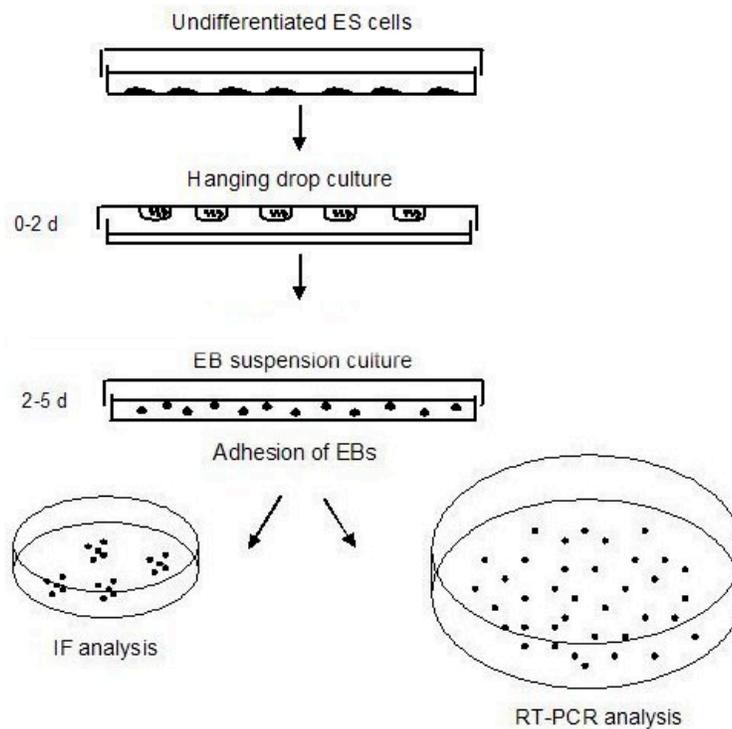

Figure 1. Schematic representation illustrating the sequential steps of KSR-based ES cell differentiation protocol (adapted from Maltsev et al., 1993).

The timeline was counted consecutively from the beginning of differentiation (see text for details). At day 0, undifferentiated ES cells were induced to aggregate by hanging drops in KSR medium. After 2 days, EBs were transferred into bacteriological Petri dishes for further three days. At day five of differentiation, EBs were induced to adhere and thus plated on either 100mm-gelatine coated-dishes for RT-PCR or 60mm gelatine-coated dishes for immunofluorescence analysis.
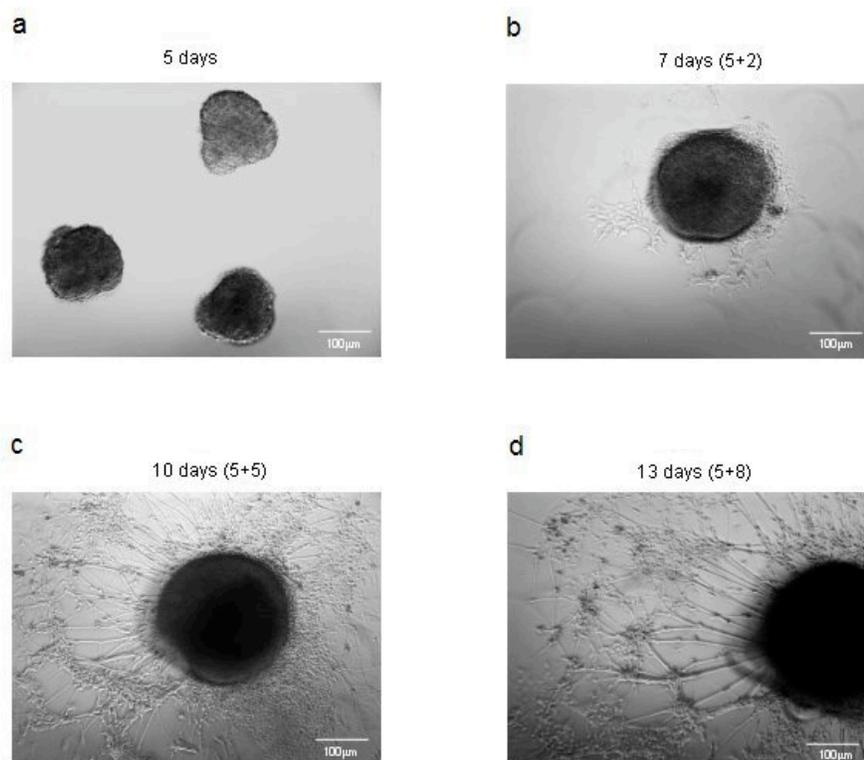
Figure 2. Time-course morphological observation of KSR-derived EBs.

Phase-contrast images (10x magnification) of RI ES cells differentiated as EBs for 5 (a), 7 (5+2, b), 10 (5+5, c) and 13 (5+8, d) days, illustrate the morphological changes and the progressive acquisition of neuronal-like morphology, that generate a complex network surrounding the EBs.

RT-PCR

Total RNAs from either undifferentiated ES cells or EBs at different stages of differentiation: 5, 7, 10 and 13 days respectively, were extracted using Trizol ultra pure reagent (Invitrogen) according to the manufacturer's instructions and subsequently incubated with DNAse RNAse-free (Ambion Austin, TX, http://www.ambion.com) to minimize genomic DNA contamination. RT Ambion Kit was used for reverse transcription. Total RNA (2 μg RNA/reaction) was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen) using random hexamers (New England Biolabs Ipswich, MA, http://www.neb.com). cDNA samples were subjected to PCR amplification using specific primers. Levels of neural markers expression were analyzed both in undifferentiated and KSR-differentiated ES cells. The PCR was carried out using standard protocols: Taq polymerase (0.1 U/reaction, Euroclone, Pero, Italy, http://www.euroclone.net), Reaction buffer (1X, Euroclone), $MgCl_2$ (1.5 mM, Euroclone), dNTPs (0.2 mM, Euroclone). Cycling parameters were as follows, denaturation at 94°C for 45 sec, annealing at 56°C for 45 sec and elongation at 72°C for 45 sec. The number of cycles was chosen to select PCR conditions on the linear portion of the reaction curve avoiding "saturation effects" of PCR. The expression of the HPRT housekeeping gene was used to normalize PCR reactions.

Primer sequences, number of cycles and the length of the amplified products are provided in Table 1.

Table 1. **Primers used for RT-PCR**

Gene	Forward primer	Reverse primer	Size (bp)*	Number of cycles
BF1	actttgagttacaacgggaccacg	aaagtaactggtctggcccgc	282	35
Brachyury	gaacctcggattcacatcgtgaga	atcaaggaaggctttagcaaatggg	160	35
ChAT	ggccattgtgaagcggtttg	tgacatgctcgggctcaggc	425	32
DAT	gtgggcttcactgtcatcctca	cccaggtcatcaatgccacga	278	35
Dlx1	caaggcggggcagctctg	gggagacgggcaggaagc	226	35
EAAT-1	ttggttcgaggacacagtgag	caggcccacgattttgtattc	504	32
En1	tcaagactgactcacagcaacccc	tgaatgtccactcggaggatt	564	32
GAD67	tacggggttcgcacaggtg	ccccaagcagcatccacat	600	32
Gbx2	cggcaacttcgacaaagc	agagagaagctctcctcctgc	200	32
GFAP	gctggaggtggagagggaca	tggcggcgatagtcgttag	456	32
HB9	acaggcggctctctatgggaca	ttccccaagagggttcgactgc	258	35
Hoxb4	cctggatgcgcaaagttcac	gtgttgggcaacttgtggtc	240	32
HPRT	cctgctggattacattaaagcactg	cctgaagtactcattatagtcaagg	369	28
Isl1	agctgtacgtgctttgttagggatg	tcttctcgggctgtttgtgga	390	32
Lmx1b	cctcagcgtgcgtgtggtc	agcagtcgctgaggctggtg	323	28
Math1	cttcctctggggggttactcg	aaactctccgtcacttctgtgg	185	35
MBP	aagcacacagcagacccaaagaa	aaggatgcccgtgtctctgt	309	32
Mlc2v	gccaagaagcggatagaaggcggg	ctgtggttcagggctcagtccttc	490	35
Nestin	cagagaggcgctggaacagagatt	agacataggtgggatgggagtgct	460	28
NF-M	gaaatggaagaaaccctcaca	ccggccttggcctctggttttgg	474	32
Nkx2.1	tactgcaacggcaacctg	gccatgttcttgctcacgtc	186	35
Nkx2.2	atgtcgctgaccaacacaaa	aaagggctcttaaggggcag	194	32
Nurr1	ccttgtgttcaggcgcagtatg	tggctgtgttgctggtagttgtg	311	32
Pax7	tgccgatatcaggagactggg	ccaggatgccatcgatgc	370	28
Pitx3	ggaatcgctaccctgacatgag	tgaaggcgaacgggaaggtc	270	35
TH	tgtcacgtccccaaggttca	gggcaggccgggtctctaagt	276	30
TpH	agacataggtgggatgggagtgct	gacatcaaggtcataccgcaac	501	32
Shh	ggaagatcacaagaaactccgaac	ggatgtgagctttggattcat	354	32

* Expected size of PCR products.

Immunocytochemistry

For immunofluorescence analysis, EBs were cultured 5 days in suspension and then plated on gelatine-coated dishes. EBs were fixed at different stages of differentiation in methanol/acetone (7:3) on ice for 20 min, or in 4% paraformaldehyde/1XPBS at room temperature (RT) for 30 min.

Staining of methanol/acetone fixed EBs: following fixation, the EBs were air dried and then re-hydrated in 1X PBS for 30 min at room temperature. Permeabilization and blocking were performed in 5% BSA/0.1% Triton X-100 (Sigma-Aldrich)/1X PBS for 15 min at RT, followed by incubation with 5% BSA in 1X PBS for 30 min at RT to minimize background. After blocking, EBs were incubated with anti-sarcomeric myosin MF-20 antibodies (1:50, monoclonal supernatant obtained from the Developmental Studies of Hybridoma

Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242), in 5% BSA /1X PBS for 2 h at RT. Following the incubation with primary antibodies, EBs were washed 3 times with PBS-0.1% Tween for 5 min at RT and further incubated with anti-mouse Cy3 secondary antibodies (1:400, Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com) in 5% BSA /1X PBS at RT for 30 min. The EBs were then washed 5 times with PBS-0.1% Tween for 5 min at RT, counterstained with DAPI (250 ng/mL, Sigma-Aldrich) and mounted with VectaShield medium (Vector Laboratories Inc., Burlingame, CA, http://www.vectorlabs.com).

Staining of PFA-fixed EBS: following fixation, EBs were washed 3 times with 1X PBS for 5min and then incubated with 10% normal goat serum (Dako Cytomation, Glostrup, Denmark, http://dakocytomation.com)/1% BSA/0.1% Triton X-100 in 1X PBS for 15 min at room temperature. The EBs were then washed 3 times in 1X PBS for 5 min and incubated with primary antibodies in 10% normal goat serum/1% BSA/1X PBS at the following working dilutions: monoclonal anti- βIII-tubulin (1:400; Sigma-Aldrich), monoclonal anti-nestin (1:10 obtained from the Developmental Studies of Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242), polyclonal anti-GFAP (1:300; Dako Cytomation), polyclonal anti-TH (1:100; Chemicon International), polyclonal anti-GABA (1:100; Chemicon International), polyclonal anti-5-HT (1:1000; Sigma-Aldrich); monoclonal anti-Islet1 (1:100 obtained from the Developmental Studies of Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242), monoclonal anti-E-cadherin (1:100, Sigma-Aldrich). Following primary antibodies incubation, EBs were rinsed 3 times in 1X PBS and further incubated with secondary antibodies: either goat anti-rabbit IgG FITC-conjugated (1:200; Santa Cruz Biotechnology, Santa Cruz CA, http://www.scbt.com) or goat anti-mouse IgG texas

red-conjugated (1:400; Molecular Probes) in 1% BSA/1X PBS for 30 min at RT. Finally, EBs were washed 4 times in 1X PBS, counterstained with DAPI (250 ng/mL, Sigma-Aldrich) and mounted in VectaShield medium (Vector Laboratories). Labeling was visualized by fluorescent illumination using an inverted microscope (DMIRB, Leica Microsystems, Wetzelar, DE, http://www.leica-microsystems.com); images were acquired on a DC 350 FX camera (Leica).

Results and Discussion

Neural and Glial Differentiation of ES Cells with KSR Method

It is well established that ES cell-derived EBs, cultured in the presence of high percentage of selected serum (15-20% FBS), spontaneously differentiate into beating cardiomyocytes [17, 18]. In these culture conditions, neuronal differentiation is achieved exclusively upon addition of specific inducing factors, such as Retinoic Acid [5, 19]. Recently, efficient neural conversion of ES cell-derived EBs has been reported to occur in serum-free conditions; however, it mainly results in lineage restricted neuronal population [13]. Here we describe a versatile and fast protocol to generate both glial cells and a wide range of neuronal subtypes from mouse ES cells, taking advantage of a chemically-defined serum free medium (KSR; see Materials and Methods) (Figure 1).

To generate size-controlled EBs, the hanging drop-based protocol was used (Figure 1). Following EBs formation and suspension culture, 5 day-old EBs were plated onto an adhesive substrate and differentiation was monitored throughout a period of 13 (5+8) days both by morphological analysis, gene expression profile and immunofluorescence. Starting from day 10 (5+5) of differentiation onward, a population of cells showing a neuron-like morphology was observed which produce a complex network surrounding the aggregates over the following 3 days (Figure 2c and d).

To confirm that those cells were indeed neurons, the expression profile of both neural

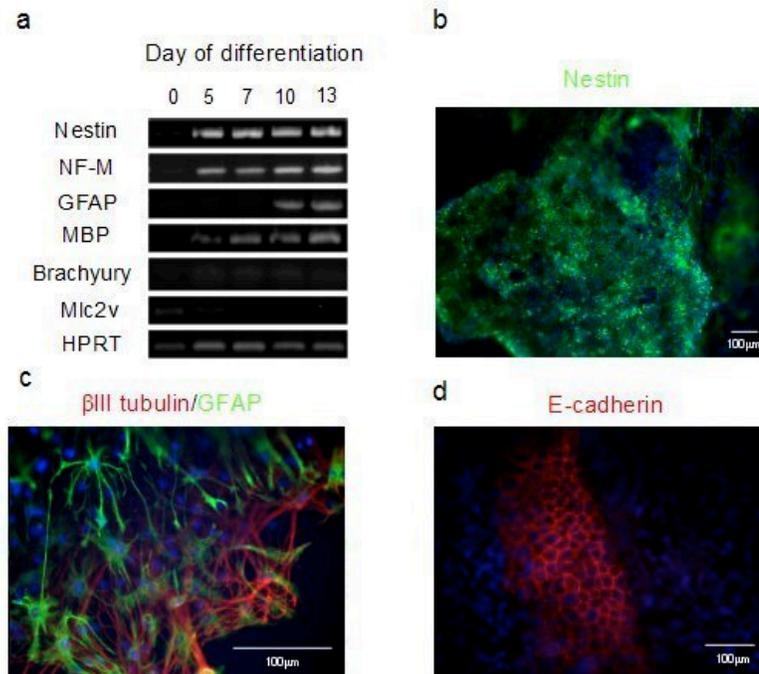precursor marker (nestin) and neuronal marker (NF-M) were analyzed, by RT-PCR (Figure 3a).

Figure 3. Mesodermal and neuroectodermal-derivatives in KSR-derived EBs.

(a) Expression profile of mesodermal and neuroectodermal specific markers, as analyzed by RT-PCR. RNAs were isolated from EBs at different days of differentiation and subjected to RT-PCR using specific primers. The timeline was counted consecutively from the beginning of differentiation: undifferentiated ES (0), EBs differentiated for 5, 7 (5+2), 10 (5+5) and 13 (5+8) days. (b-d) Immunofluorescence analysis performed on KSR-derived EBs. (b) Nestin/DAPI staining of 7-day-old EBs showed large areas of immunoreactive cells (10x magnification). (c) GFAP/βIII tubulin double staining of 13-day-old EBs (40x magnification) revealed the presence of both astrocytes (green) and neurons (red) in KSR-derived EBs. Cells were counterstained with DAPI (blu). (d) E-cadherin/DAPI staining of 10-day-old EBs showed areas of E-cadherin-ir+ cells (20x magnification) indicating that ectodermal tissue was properly formed.

Indeed, expression of both nestin and NF-M was already detectable at day 5 of differentiation, thus suggesting that a rapid differentiation of ES cells into neural lineages occurred in these culture conditions. Accordingly, immunofluorescence analysis showed that 100% of EBs analyzed were immunoreactive to nestin (Figure 3b). Moreover, staining with βIII-tubulin antibodies revealed the presence of large clusters of post-mitotic neurons, eventually forming a dense network of neurites in 13-day-old EBs (Figure 3c). Furthermore, spontaneous differentiation of astrocytes occurred in KSR-derived EBs, as shown by both RT-PCR and immunofluorescence analysis (Figure 3a and c). Indeed, GFAP transcripts were detected starting from day 10 (5+5) of differentiation; accordingly,

100% of the 13 day-old EBs that had been analyzed, stained positive with anti GFAP antibodies (Figure 3a and c). Conversely, MBP-ir+ cells were never detected in our cultures, thus suggesting that oligodendrocytes were undetectable by immunofluorescence in KSR-derived EBs; although, expression of this marker was observed, at low levels, by RT-PCR (Figure 3a).

Our data thus show that KSR-derived EBs undergo a rapid and progressive differentiation toward the neuronal and glial lineages. In sharp contrast, neither βIII-tubulin nor GFAP-immunoreactive cells were ever detected in EBs differentiated under standard serum supplemented conditions [20] (Figure 4c,e,g,i).
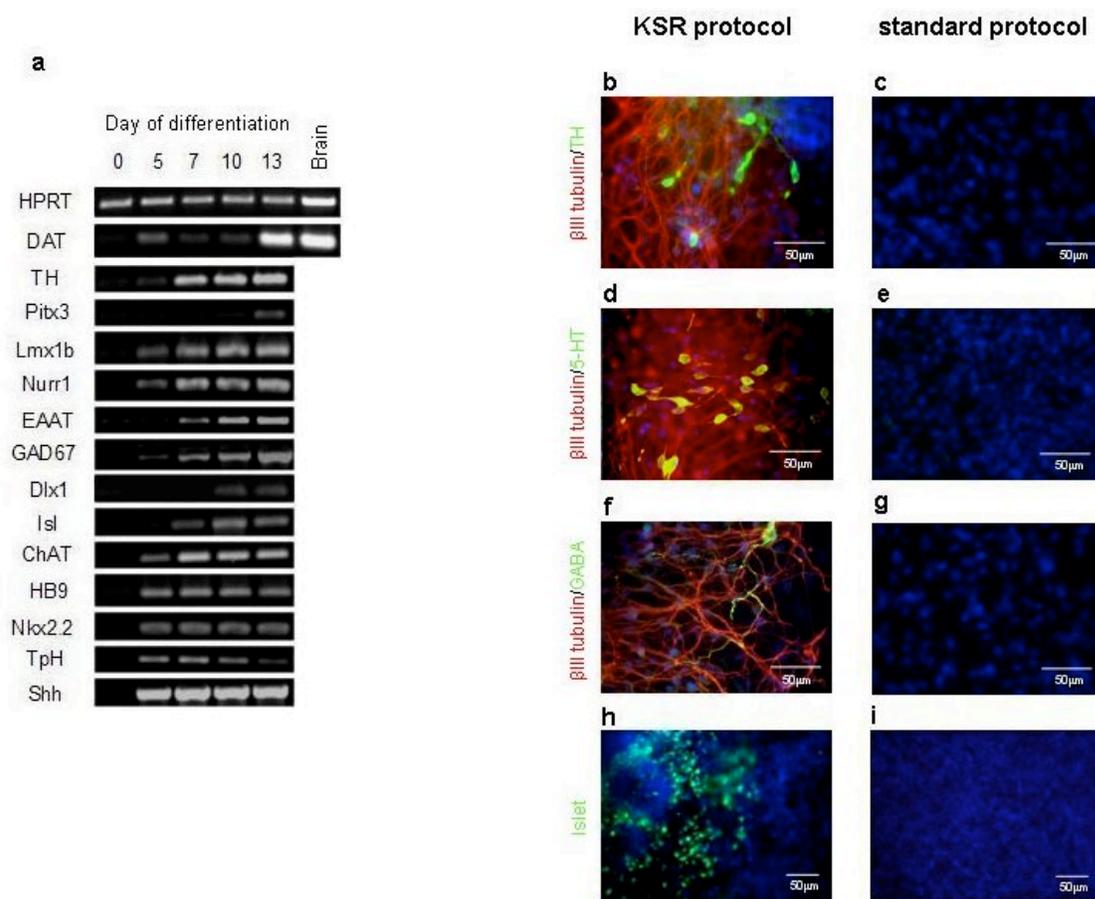
Figure 4. Neuronal fate specification of KSR-derived EBs.

(a) RNAs were isolated from EBs at different days of differentiation and subjected to RT-PCR using specific primers. RT-PCR analysis of neural-specific markers showed the presence of multiple neuronal subtypes such as GABA, serotonin, DA, and motor neurons starting from days 5-7 of the *in vitro* differentiation. The timeline was counted consecutively from the beginning of differentiation: undifferentiated ES (0), EBs differentiated for 5, 7 (5+2), 10 (5+5) and 13 (5+8) days. RNAs from adult mouse brain were used as positive control. According to RT-PCR results (a) double staining of βIII tubulin with TH (b), serotonin (5-HT, d), GABA (f) antibodies (63x magnification) and Islet antibodies (g) (40x magnification), showed a marked presence of mid-hindbrain and spinal cord neurons in EBs differentiated by KSR protocol. In sharp contrast, we never observed positive staining using the same antibodies in EBs differentiated under standard serum supplemented conditions (c, e, g, i).

Having shown that KSR-derived EBs efficiently differentiate into neuroectodermal-derived cells, we asked whether induction of neuronal markers was accompanied by the presence of non-neural ectodermal tissue. To this end, immunofluorescence analysis was performed using anti E-cadherin antibodies, staining the non-neural ectodermal cells. Indeed, 100% of EBs analyzed showed areas of E-cadherin-immunoreactive cells (Figure. 3d), thus indicating proper formation of the ectodermal lineage in these culture conditions.

We thus went on to evaluate whether efficient neural differentiation resulted in impaired differentiation of mesodermal tissues in KSR-derived EBs. To this end, the expression profile of both the pan-mesodermal marker *Brachyury* and the cardiac-specific marker Mlc2v, was analyzed by RT-PCR (Figure. 3a). Both genes are expressed in a time-dependent manner during EBs differentiation in serum-containing medium ([20, 21] and data not shown); in sharp contrast, expression of *Brachyury* and Mlc2v was hardly detected in KSR-derived

EBs, thus suggesting that mesodermal induction and differentiation was impaired in these culture conditions. Accordingly, a small percentage (10%) of KSR-derived EBs showed few MF-20-ir^{+} cells (data not shown). Taken together, our results indicate that the KSR-protocol favors differentiation into neuro-ectodermal lineages.

Generation of Multiple Neuronal Subtypes in KSR-Derived EBs

Previous reports have shown that multiple neuronal subtypes can be derived from mouse ES cells either by co-culture with stromal cells [6, 9] or by induction with specific inducing factors [5, 19]. Furthermore, very recently, the generation of ES-derived neurons has been reported to occur in serum free cultures [13], or in adherent culture in presence of specific growth factors [14]. Worth noting, both protocols result in highly efficient neuronal induction and differentiation of ES cells; nevertheless, lineage restricted sub-neuronal population was obtained.

Having shown that KSR-derived EBs underwent rapid and efficient neuronal differentiation, we went on to assess whether different neuronal subtypes could be derived in these culture conditions. To this end, the time-dependent expression profile of specific neuronal markers was analyzed by RT-PCR and, furthermore, immunofluorescence analysis was performed using anti-βIII-tubulin antibodies in combination with markers of different neuronal subtypes. We first evaluated the presence of neurons expressing the tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine biosynthesis. Interestingly, almost 100% of 13 day-old EBs analyzed showed clusters of TH-immunoreactive neurons (Figure 4b). Consistent with these results, the dopamine transporter (DAT) was highly expressed (Figure 4a); thus revealing the presence of dopaminergic neurons in KSR-derived EBs. Furthermore, the presence of serotonin (5-HT) immunoreactive cells within the cultures indicated that serotonergic neurons were also present in 100% of EBs analyzed (Figure 4d). Accordingly, the serotonin biosynthetic enzyme tryptophan

hydroxylase (TpH) was highly expressed (Figure 4a). Worth noting, GABA-ir^{+} cells were observed only in 25% of the EBs analyzed (Figure. 4f), thus revealing the presence of rare GABAergic neurons, that is consistent with the expression of the glutamate decarboxilase GAD67 gene, detected by RT-PCR (Figure 4a). Furthermore, the excitatory aminoacid transporter EAAT transcript was also detected (Figure 4a); thus suggesting that glutamatergic neurons were also present in these cultures. Finally, 50% of the EBs analyzed showed areas of Isl1-ir^{+} cells thus indicating the presence of cholinergic neurons (Figure 4h). The generation of these neuronal sub-types was consistent with the presence in these cultures of both mid-hindbrain (En1, Nurr 1, Lmx1b, Pitx3 and Nkx2.2), spinal cord (HB9, ChAT) and forebrain (Dlx1) specific transcripts (Figures 4a, 5).

Worth noting, we never detected immunoreactive cells using TH (Figure 4c), serotonin (Figure 4e), GABA (Figure. 4g), and Isl1 (Figure 4i) antibodies in EBs differentiated under standard serum supplemented conditions.

It is well known that early exposure to Shh during ES cell differentiation induces the expression of ventral markers and is required for enriching in dopaminergic, serotonergic and cholinergic neurons [22, 23]. Based on these considerations and on our results, we evaluated the expression of Shh in KSR-derived EBs cultures, by RT-PCR. Indeed, expression of Shh was highly induced already in 5 day-old KSR-derived EBs, persisting at high levels up to day 13 (Figure 4a); thus leading to hypothesize that early endogenous expression of Shh might reflect the high default prevalence of ventral neuronal subtypes observed in these cultures.

Brain regional markers expressed by ES cell-derived neurons in KSR culture condition

With the aim to better characterize the nature of the ES cell-derived neurons in KSR culture conditions, we analyzed the expression of rostral-caudal CNS markers, by RT-PCR. Interestingly, KSR-differentiated EBs express the spinal cord marker Hoxb4, the hindbrain marker Gbx2, the

midbrain marker En1 as well as the forebrain marker Dlx1, whereas expression of telencephalic markers BF1 and Nkx2.1 were barely detected (Figure 5). These results indicate that KSR-differentiated ES cells give raise to a variety of neuronal cell types along rostro-caudal axis, with the exception of the rostral most cells of the CNS. Interestingly, Watanabe and colleagues have recently shown that a marked increase in telencephalic neurons can be obtained upon neuronal differentiation of ES cell in similar serum-free culture conditions; however, EBs were generated by spontaneous aggregation in suspension culture and additional inducing factors were used that might explain the different neuronal subtypes generated. Indeed, ES cells were cultured in the presence of GMEM supplemented with N2 and, in addition, the EBs were plated on poly-D-lysine, laminin and fibronectin coated dishes from day 5 of differentiation [13].
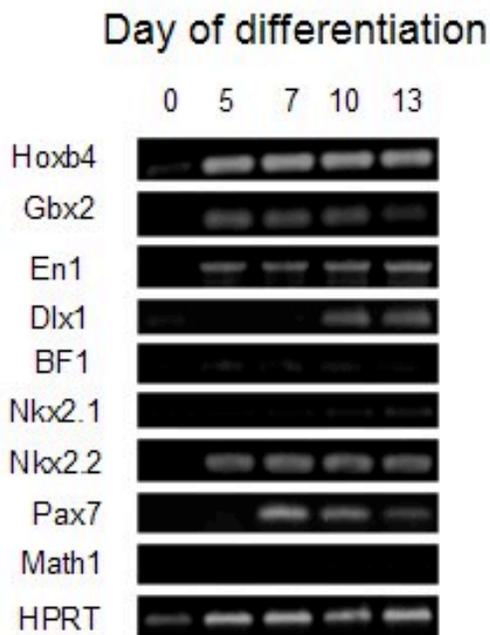

Figure 5. Positional identity of KSR-derived neurons.

RNAs were isolated from EBs at different days of differentiation and subjected to RT-PCR using specific primers. The timeline was counted consecutively from the beginning of differentiation: undifferentiated ES (0), EBs differentiated for 5, 7 (5+2), 10 (5+5) and 13 (5+8) days. Expression profile of rostro-caudal (BF1, Nkx2.1, Dlx1, En1, Gbx2, Hoxb4) and dorso-ventral (Math1, Pax7, Gbx2, Nkx2.2, Dlx1, Nkx2.1) markers revealed the presence of a variety of dorso-ventral as well as rostro-caudal neurons within the cultures.

Finally, to further characterize the neuronal subtypes generated by the KSR protocol, we examined the expression profile of positional markers along the dorsal-ventral axis in KSR culture conditions (Figure. 5). The ventral markers Nkx2.2, Dlx1 and Gbx2 were all expressed in KSR-derived EBs. Moreover, the dorsal marker Pax7 was expressed in KSR culture conditions starting from day seven, whereas, in sharp contrast, expression of Math1 marker, which identifies the dorsal-most interneuron progenitors, was not detected. These results suggest that KSR-derived EBs have the competence to differentiate into a variety of dorsal and ventral cells of the CNS except for the dorsal most cells of the neural tube.

Conclusions

In this study, we describe a rapid and versatile method to generate a wide range of neural subtypes from mouse ES cells. Indeed, we have shown that neurons expressing positional markers characteristic of midbrain, hindbrain and spinal cord can be easily and rapidly generated. Moreover, ES cells remain amenable to dorso-ventral neural patterning and may thus serve as a universal source for neuronal subtype-specific differentiation. Finally, in KSR-based ES cell differentiation protocol the temporal expression profile of genes involved in early neuronal commitment and differentiation (i.e. nestin, TH, ChAT, Nurr1, GAD67, Pitx3), gliogenesis (GFAP) as well as of genes involved in anterior-posterior and dorso-ventral patterning (i.e. Dlx1, Hoxb4, Pax7 and Nkx2.2) generally mimicked that observed in mouse embryo [9]. In this respect, the reported protocol would be very useful to improve our knowledge of the molecular mechanisms underlying neuronal differentiation in mammalian cells. Furthermore, we believe that, upon addition of defined cocktail of growth factors and/or extra-cellular matrix components, this protocol might be useful to obtain lineage restricted neuronal population, thus representing a valuable source of neuronal subtypes for transplantation

experiments in animal models of neurodegenerative diseases.

Acknowledgements

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