Associazione di **B**iologia **C**ellulare e del **D**ifferenziamento

Stem Cells, Development and Regenerative Medicine

Programme & Abstracts

Turin, 4-6 May 2012

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Organisers

Antonio Musarò (Chair) - Sapienza University of Rome

Giorgio Merlo (co-Chair) - Molecular Biotechnology Center, University of Torino

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PROGRAMME

Friday, 4 May

13:00 **Registration**

14:00-15:30 **Neurogenesis and Neural Development**

Chair: Ferdinando Di Cunto (Turin)

Nicoletta Carucci (Rome)

Region-specific function and regulation of Retinoic Acid (RA) signalling in neural progenitors

Federico Luzzati (Turin)

Neuronal genesis in the striatal parenchyma during progressive degeneration

Chiara Rolando (Turin)

Distinct roles of NOGO-A and NOGO receptor 1 signaling in the homeostatic regulation of adult Neural Stem Cell activity and in neuroblast migration

Gabriella Sarò (Turin)

The role of ArhGAP15, a negative regulator of the small-GTPase Rac1, in neuronal migration and neuritogenesis during cortical development

15:30-16:00 **Coffee break**

16:00-17:50 **Development and Disease**

Chair: Antonio Musarò (Rome)

Caterina Missero (Naples)

p63 regulation of gene expression programs in skin development and disease

Nadia Lo Iacono (Milan)

Pin1 is a Dlx5 target gene that modulates ΔNp63α protein stability during limb development

Elisa De Luca (Turin)

Characterization of vascular smooth muscle cells in zebrafish

Giulia Garaffo (Turin)

The homeodomain gene Dlx5 controls peripheral olfactory differentiation and axonal connection, via complex protein-coding and microRNA regulations

Anna Cariboni (London, United Kingdom)

Failure of GnRH neuron migration and survival in mice lacking SEMA3A and VEGF164 signalling via Neuropilins

Free Evening

Saturday, 5 May

9:00-9:55 **Invited Speaker**

Vania Broccoli (Milan)

Speeding up genetic cell reprogramming: the direct shortcut

10:00-10:40 **Stem Cells, Development, Differentiation (1)**

Chair: Enzo Calautti (Turin)

Matteo Perino (Milan)

Direct conversion of mouse and human fibroblasts into functional dopaminergic neurons

Daniela Privitera (Milan)

Development of pantotenate kinase associate neurodegeneration disease model by direct reprogramming of patients' fibroblasts

10:40-11:10 **Coffee break**

11:10-12:30 **Stem Cells, Development, Differentiation (2)**

Chair: Enzo Calautti (Turin)

Sharmila Fagoonee (Turin)

The RNA-binding protein, ESRP1, is a novel regulator of pluripotency in mouse embryonic stem cells

Silvia Pellegrini (Milan)

Human induced pluripotent stem cells (hiPS) as a source of insulin-producing cells

Alessandro Rosa (Rome)

Generation of new tools and iPS cell-based *in vitro* model systems to study Amyotrophic Lateral Sclerosis (ALS)

Ilda Theka (Milan)

Establishing a fast method for the generation of functional dopaminergic neurons from human iPS cells

12:30-13:10 **Stem Cells and Cancer**

Chair: Sara Cabodi (Turin)

Caterina La Porta (Milan)

Senescent cells in growing tumors: population dynamics and cancer stem cells

Elisabetta Ercole (Turin)

Human medulloblastoma stem-like cells: comparison between cancer stem cells from medullospheres and basal cell lines

13:10-14:20 **Lunch**

14:20-15:15 **Invited Speaker**

Maria Pia Cosma (Barcelona, Spain)

Somatic cell reprogramming as a mechanism for tissue regeneration

15:20-16:40 **Signaling in Development**

Chair: Massimo Santoro (Turin)

Alessandra Costa (Rome)

Exploiting Vasopressin signaling in muscular atrophy and dystrophies

Irene Franco (Turin)

Class II PI3K-C2α: a novel regulator of vesicular trafficking at the base of the primary cilium

Anna Musto (Naples)

Dies1: a new player of BMP4 signalling in ESCs

Giusy Tornillo (Turin)

Role of p130Cas in the control of epithelial cell commitment and differentiation in the mammary gland

16:40-19:00 **Poster Session and Coffee Break**

20:00 **Social dinner at restaurant "La Pace"**

Sunday, 6 May

9:30-11:00 **Stem Cells and Regeneration**

Chair: Giorgio Merlo (Turin)

Viviana Costa (Rome)

A common progenitor gives rise to hepatic epithelial and mesenchymal derivatives: new insights in development, regeneration and liver fibrosis

Letizia De Chiara (Turin)

Murine germline-cell derived pluripotent stem cells differentiated into tubular-like renal epithelial cells can protect from renal ischemia/reperfusion injury

Gabriella Ranaldo (Novara)

A novel iPSC-based strategy to correct the bleeding phenotype in hemophilia A

Diego Zanolini (Novara)

FVIII expression and secretion in bone marrow cells capable of correcting bleeding in hemophilia A mice

11:00-11:30 **Coffee break and departure**

ABSTRACTS

Oral Presentations

in alphabetical order
(presenting authors are shown underlined)

Speeding up genetic cell reprogramming: the direct shortcut

V. Broccoli[1], M. Caiazzo[1], M.T. Dell'Anno[1], F. Ungaro[1], S. Curreri[2], D. Leo[2], R. Gainetdinov[2], G. Pezzoli[3], A. Dityatev[2]
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Having access to human neurons for regenerative therapies and understanding diseases has been prohibited for long time. However, genetic technologies of cell reprogramming have widen opened this possibility by in vitro differentiation of fibroblast-derived iPS cells. However, a new method of cell reprogramming has become available which convert fibroblasts directly into functional neurons without passing through a pluripotent stem cell stage (iPSCS). This technology has some significant advantages since it is very fast and efficient and can be employed with human adult fibroblasts as well. Recently, we found a minimal set of three transcription factors (Mash1, Nurr1 and Lmx1a) able to efficiently convert mouse and human fibroblasts into functional dopaminergic neuronal (iDAN) cells. Molecular and transcriptome studies showed iDAN cells to recapitulate gene expression of their brain homolog neurons to large extent while lacking expression of other monoaminergic neuronal subtypes markers. Strikingly, iDAN cells showed spontaneous electrical activity organized in regular spikes consistent with the pacemaker activity featured by brain DA neurons. Furthermore, iDAN cells express D2 autoreceptors and their activity is regulated by the D2/3R agonist quinperole. The three factors were able to elicit DA neuronal conversion in adult fibroblasts from healthy donors and Parkinson's disease patients. Importantly, when transplanted in rats unilaterally lesioned with 6-OHDA, mouse iDAN cells were able to rescue drug-induced locomotor impairment over long time. In order to strength and expand iDAN cell therapeutic potential, we devised a remote control system to modulate activity of the transplanted iDAN cells. This method relays on a ligand molecule, which crosses efficiently the blood-brain barrier and is safe and inert for the entire living organism. With this system, we foresee to modulate iDAN cell activity in vivo to regulate their therapeutic effects depending by the patient's needs.

Failure of GnRH neuron migration and survival in mice lacking SEMA3A and VEGF164 signalling via Neuropilins

A. Cariboni[1,5], K. Davidson[2], V. Andre'[5], F. Stossi[4], R. Maggi[5], S. Rakic[1], J.G. Parnavelas[1], C. Ruhrberg[2]
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Reproduction in mammals is regulated by gonadotropin-releasing hormone (GnRH) neurons, which originate in the nasal placode and migrate along vomeronasal axons to enter the brain. Failure of these neurons to migrate or survive results in infertility, with genetic and molecular mechanisms not fully understood. By combining the analysis of genetically altered mice with in vitro models, we show here that GnRH neuron development relies on two distinct neuropilin (NRP)-mediated signalling pathways. Firstly, we found that the vascular endothelial growth factor VEGF164 activates its receptor NRP1 to promote the survival of migrating GnRH neurons. Secondly, we showed that the semaphorin SEMA3A is essential to guide vomeronasal axons and, therefore, GnRH neurons into the brain, by signalling through its classical receptor NRP1 and, unconventionally, NRP2. Consequently, the combined loss of SEMA3A and VEGF164 precludes the development of GnRH neuron system, demonstrating the essential role of NRP signalling in the control of reproduction.

Region-specific function and regulation of Retinoic Acid (RA) signalling in neural progenitors

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RA is a vitamin A derivative that plays a crucial role in the specification of the posterior nervous system (hindbrain and spinal cord) during early stages of vertebrate development. By contrast, at later stages, while continuing to be implicated in posterior neural development, RA signalling is also required for forebrain development. This suggests that epigenetic regulatory mechanisms locally modulate the response of neural progenitor cells (NPCs) to RA, in order to achieve appropriate, position-dependent, effects. Very little is known, however, on the region-specific mechanisms controlling RA signalling during neural development.
We have studied the response of NPCs derived from E13.5 mouse cortex (Ctx), lateral ganglionic eminence (LGE) and spinal cord (SC) to RA. Ctx, LGE and SC NPCs all expressed the RA receptors RARa, RARb and RARg and responded to exogenous RA upregulating the RA target genes RARb and Dhrs3. Thus, the RA signalling pathway is functional and elicits a transcriptional response in NPCs from different neural areas. However, RA activated specific transcriptional programmes in Ctx, LGE and SC NPCs. In particular, RA treatments upregulated HoxB4, HoxB6, HoxB8 and HoxB9 in SC NPCs, Dlx2 and Six3 in LGE NPCs, while none of these genes were upregulated in Ctx NPCs.
We are investigating the molecular mechanisms modulating the response of Ctx, LGE and SC NPCs to RA. RARa, RARb and RARg are thought to act redundantly in transduction of RA signalling, but they may also have specific, non-redundant functions. Therefore, we are generating NPCs lines where individual RARs are abrogated, to address whether the differential response of region-specific NPCs to RA involves context-dependent regulation of specific receptors. Furthermore, we are performing ChIP assays with RA-treated NPCs using anti-RAR antibodies, to determine whether the activation of region-specific transcriptional responses by RA reflects context-dependent binding of RARs to target genes.

Somatic cell reprograming as mechanism for tissue regeneration

M.P. Cosma
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Spontaneous cell fusion between two cells of different lineages can originate new hybrid cells that have different features from the original parent cells. If one of the fusing parent cells is highly plastic, such as a stem cell, and the other is a somatic cell, their fusion can be followed by reprogramming events that generate new hybrid pluripotent cells. However, whether cell-fusion-mediated reprogramming can occur in vivo in higher vertebrates, and what are the molecular mechanisms and genes that drive the reprogramming, remain to be defined. We have shown that activation of the Wnt/beta-catenin signalling pathway enhances reprogramming of somatic cells after their fusion with embryonic stem cells. We are currently dissecting out the gene networks and studying the mechanisms of in-vivo somatic-cell reprogramming, to determine whether reprogrammed hybrids have the potential to differentiate and regenerate tissues.

Exploiting Vasopressin signaling in muscular atrophy and dystrophies

A. Costa, B.M. Scicchitano, S. Adamo
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Arginine-Vasopressin (AVP) is a neurohypophyseal hormone able to induce differentiation in myogenic cell lines (L5 and L6) and primary satellite cells. V1aR is the only AVP receptor expressed in skeletal muscle. By interacting with V1aR, AVP activates phospholipases C and D, increases cytosolic Ca2+ concentrations and regulates cAMP levels. The AVP-dependent increase in cytosolic calcium activates CaMK and calcineurin pathways resulting in the formation of multifactor complexes on the promoter of muscle specific genes. Our previous data demonstrate that V1aR expression is modulated in muscle regeneration and that the stimulation of AVP signaling strongly enhances regeneration of cardiotoxin injured muscle. In an experimental model of muscular atrophy induced by TNF over-expression, stimulation of AVP pathways counteracted the negative effects of TNF, evaluated by morphological and molecular analysis. The expression levels of early regeneration markers such as Pax7 and MyoD were up-regulated by TNF whereas late differentiation markers such as myogenin and MHC were down-regulated suggesting an impairment of regeneration. This effect was counteracted by V1aR overexpression. The positive effects of V1aR on muscle homeostasis are due to the promotion of the calcinuerin-IL-4 pathway and by the inhibition of atrophic genes expression mediated by FOXO phosphorilation. Indeed we demonstrated that high levels of TNF in muscle stimulates protein degradation via the Akt-dependent pathway and this effect is counteracted by local V1aR overexpression. By all the above we are analyzing the effects of AVP signaling stimulation in mouse models of muscular dystrophies. Preliminary data demonstrate that stimulation of AVP-dependent pathways ameliorate inflammation and regeneration processes. This study highlights a novel in vivo role for the AVP-dependent pathways which may represent a potential gene therapy approach for many diseases affecting muscle homeostasis.

A common progenitor gives rise to hepatic epithelial and mesenchymal derivatives: new insights in development, regeneration and liver fibrosis

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Tissues of the adult organism maintain homeostasis and respond to injury by means of progenitor/stem cell compartments capable to give rise to the appropriate progeny . In organs composed by cell types of different embryological origins (e.g. the liver), the normal tissue turnover may in theory involve different stem/precursor cells able to respond coordinately to physiopathological stimuli. The unique feature of the liver to regenerate acutely (e.g., following partial hepatectomy) has been mainly ascribed to proliferation of terminally differentiated parenchymal liver cells (hepatocytes and colangiocytes) in response to mitogenic stimuli. However, it has also been shown that, in response to chronic injury or to toxics inhibiting hepatocytes proliferation, a hepatic progenitor cells compartment (HPCs) giving rise to hepatocytes and cholangiocytes is activated. Regarding the Hepatic Stellate Cells (HSC, named also Ito cells or fat storing cells), a unique and elusive peri-sinusoidal cell implicated with the origin of liver fibrosis, the precursor compartment guaranteeing their turnover in adult organ is yet unveiled.
We show here that both epithelial and mesenchymal liver cell types including hepatocytes, cholangiocytes and HSCs arise from a common progenitor.
In fact, orthotopic transplantation of cells of clonal origin generated epithelial liver specific and also, surprisingly, hepatic mesenchymal derivatives suggesting the existence of a "bona fide" organ-specific meso-endodermal precursor cell and thus profoundly modifying current models of progenitor commitment. Heterotopic transplantation and in in vitro experiments suggest that the precursor fate is also controlled in a cell autonomous fashion.

Murine germline-cell derived pluripotent stem cells differentiated into tubular-like renal epithelial cells can protect from renal ischemia/reperfusion injury

L. De Chiara[1], S. Fagoonee[1], S. Bruno[1,2], A. Ranghino[2], F. Barbieri[1], G. Camussi[1,2], L. Silengo[1], F. Altruda[1]
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Germline-cell Derived Pluripotent Stem Cells (GPSCs) are ES-like cells that are able to self-renew and differentiate into derivatives of all three germ layers. The purpose of this study is to investigate the capacity of GPSCs to differentiate into renal tubular cells both in vitro and in vivo. We hence devise a new protocol to induce renal epithelial differentiation, consisting in the addition to GPSCs culture medium of different growth factors, required during renal development. We demonstrated the expression of renal markers and the in vitro functionality of differentiated renal cells, and now we are investigating the capacity of these cells to integrate/repopulate the renal parenchyma after ischemia/reperfusion injury. To assess whether GPSCs-derived renal epithelial cells could colonise mouse kidney, we performed renal ischemia/reperfusion injury and injected Vybrandt CFSE-labelled differentiated cells in mice tail. Blood urea-nitrogen (BUN) and creatinine level were assessed 48h after treatment. Interestingly, in mice treated with GPSCs-derived renal cells BUN and creatinine levels are significantly lower, compared to PBS-injected ischemised mice. In these mice there is also lower compromission of renal parenchyma, infact the renal cortex of mice treated with differentiated cells shows less apoptotic cells and necrotic tubules compared to PBS-injected ischemised mice. We are currently evaluating the role of GPSCs-derived renal epithelial cells in a model of renal chronic damage. Mice were sacrificed after 6 weeks post-ischemia; mice treated with differentiated cells show less number of tubular dilatation and less fibrosis development comparted to mice injected only with PBS. These preliminary datas highlight that GPSCs-derived renal cells can protect against both acute and chronic damage.

Characterization of vascular smooth muscle cells in zebrafish

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Several early and adult cardiac diseases are associated with defects in heart development. Due to its optical clarity that allows easy observation of the internal organs during their morphogenesis zebrafish is an ideal model to study mechanisms underlying heart development. The heart is the first organ to form and function during zebrafish embryogenesis. It is composed of two chambers with an outer muscular layer, an inner endothelial layer and an outflow tract (OFT) covered by vascular smooth muscle cells (vSMCs). Since the origin of vSMCs is still poorly understood, we trace in vivo origin of these cells in zebrafish embryos using the Cre-loxP strategy to study spatial-temporal cell tracing. Through the Tol2 transgenesis we have generated the Cre driver line cmlc2::creERT2, in which the temporal control of Cre recombination is achieved by the addiction of the active metabolite of tamoxifen (4OHT) and the spatial regulation of Cre expression is obtained through the use of cmlc2 promoter, exclusively expressed in differentiated myocardial cells. When crossed with the red (DsRed) to green (GFP) reporter line, Cre activation induces the irreversible loss of red fluorescence and the constitutive expression of green. This allows following the green marked cells also after heart formation. After treatment with 4OHT at early developmental stages we have detected at 4dpf a group of green cells in the distal OFT that localize in the region positive for *tgln*, a specific marker of SMCs in zebrafish. This finding suggests that a small population of vSMCs of the OFT could result from the differentiation of a cardiogenic precursors or derive from the transdifferentiation of atrial or ventricular cardiomyocytes occurring after the primary heart morphogenesis. Since a consistent number of congenital heart diseases are associated with an abnormal OFT morphogenesis, a better understanding of the mechanisms that regulate OFT development could be useful to treat such pathologies.

Human medulloblastoma stem-like cells: comparison between cancer stem cells from medullospheres and basal cell lines

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The study of cancer stem cells (CSC) is an emerging and interesting field that concerns tumor genesis and relapse. A more detailed analysis of CSC may further unravel mechanisms related to resistance to therapies through self renewal of this particular cancer cell population. Medulloblastoma (MDB) is a highly aggressive and invasive paediatric tumour of central nervous system (CNS) belonging to PNET category. MDB is therefore a malignant, metastatic cancer and its prognosis is often poor.
A human MDB cell line, named UW228, was grown both in adhesion standard conditions and with a home made serum-free medium designed to aggregate and maintain cells in spheres called medullospheres (MS). To characterise this two cell populations a morphologic, ultrastructure and cytofluorimetric assays were performed up to fourth passage. MS showed a different shape, increasing immature features and higher stem cell markers levels such as CD133, CD44, Nestin, Nanog, Musahi-1 and Oct-4. Morevover a cytoblock technique for cell culture to obtain a tissue microarray for immuohistochemical analysis was customized. A proteomic approach consisting in 2-DE and MALDI-TOF analysis is also in progress to determine the difference between MDB cell line and medullospheres.
In conclusion our preliminary data and cell culture conditioning based on low attachment flask and home made formulation of medium may allow to study other tumor cell lines in peculiar growing conditions possibly more mimicking in vivo tumor expansion and resistance to treatments.

The RNA-binding protein, ESRP1, is a novel regulator of pluripotency in mouse embryonic stem cells

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Embryonic stem (ES) cells provide a unique opportunity to study early development and tumorigenesis, especially because an ES cell-like transcriptional program has been found active in several tumors. Thus, understanding how pluripotency is regulated has become imperative. While much is known on the transcription factors regulating the core pluripotency factors, there is little detailed understanding of the role of RNA binding proteins in this process. In order to screen for new RNA binding proteins involved in pluripotency maintenance, we bioinformatically analysed our recently performed time-course gene expression profiling of differentiating mouse pluripotent stem cells. Stem cell-specific conserved co-expression analysis of genes rapidly downregulated upon differentiation revealed the epithelial cell-specific regulatory protein 1 (ESRP1) as candidate pluripotency gene. Surprisingly, downregulation of ESRP1 in mouse ES cells resulted in highly efficient self-renewal and proliferative capacities as well as increased expression of pluripotency factors. Concordingly, ESRP1 knockdown ES cells showed impaired differentiation in the early stages of embryoid body formation. We are currently studying the mechanisms by which ESRP1 may be involved in the post-transcriptional processing of the core pluripotency factors, and we propose a new role for ESRP1 as a regulator of pluripotency.

Class II PI3K-C2α: a novel regulator of vesicular trafficking at the base of the primary cilium

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The class II phosphoinositide 3-kinase PI3K-C2α is a protein of the early endocytic compartment and of the trans-Golgi network (TGN) involved in several processes of vesicular trafficking, PI3K-C2α exerts this function through the direct interaction with clathrin and the catalytic activity towards different lipid substrates involved in membrane dynamics,
Through the generation of a Pik3c2a knock-out mouse strain, we discovered that PI3K-C2α was fundamental during embryonic development and that its loss principally affected the structure and function of a cellular organelle deeply involved in development: the primary cilium.
Analysis of Pik3c2a deficient embryos revealed that primary cilia were shorter and swollen and displayed a defect in accumulating signaling proteins, such as Smo and Polycystin-2. Consistently, the mutation conferred features of ciliopathy: homozygous mutant embryos died at midgestation dysplaying laterality defects and impaired Hedgehog signaling, while heterozygous adults showed renal cysts susceptibility after kidney injury.
In primary mouse embryonic fibroblasts (MEFs), PI3K-C2α was highly enriched at the basal body of primary cilia and the absence of the protein in Pik3c2a knock-out MEFs specifically caused a reduction of vesicular trafficking at the cilium base.
All these data indicate that PI3K-C2α is required for the correct exchange of structural proteins and signaling molecules between the cilium compartment and the cytoplasm and suggest PI3K-C2α as a novel regulator of vesicular trafficking at the base of the primary cilium.

The homeodomain gene *Dlx5* controls peripheral olfactory differentiation and axonal connection, via complex protein-coding and microRNA regulations

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During embryonic development, the olfactory sensory system is formed by the coordinated morphogenesis and differentiation of the peripheral olfactory epithelium (OE) and of the anterior forebrain. Immature olfactory receptor neurons (ORN) elongate their axons to contact and form synapses with projection neurons of the olfactory bulb (OB). Axon elongation is accompanied by migration of the GnRH+ neurons, a process specifically impaired in the Kallmann's syndrome, a condition leading to anosmia and central hypogonadism.
Axon elongation and connection are controlled by signals and interactions between the ORN and adjacent cells (via morphogens, guidance molecules, ECM..), but recent evidence identify specific transcription factors (i.e. Dlx5, Fez, Emx2) as master regulators of this process. Starting point of our study is the analysis of Dlx5 mutant mice, in which we observed a differentiation delay of ORN and a failure of ORN axons to connect with the OB, during the development.
We have combined expression profiling of genes and miRNAs, comparing normal and Dlx5-/- OE, with conserved co-expression network (CLOE) and with prediction of Dlx5 putative binding sites on the genome. We found 80 downregulated coding genes and 6 downregulated miRNAs. Based on the current results, the emerging hypothesis is that Dlx5 controls differentiation and maturation of ORN via the action of two miRNAs (miRNA-200 and miRNA-9, which in turns regulates the transcription factor Foxg1), while controls axon connection via a set of protein-coding genes. We are functionally testing a number of coding genes in a transgenic strain of zebrafish, to assess their role in olfactory axon elongation, guidance and connection.
The relevance of unveiling the molecular basis of this developmental process lies in the peculiarity of the peripheral olfactory system to regenerate cells and connection both physiologically and after a lesion.

Senescent cells in growing tumors: population dynamics and cancer stem cells

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Tumors are defined by their intense proliferation, but sometimes cancer cells turn senescent and stop replicating. In the stochastic cancer model in which all cells are tumorigenic, senescence is seen as the result of random mutatations, suggesting that it could represent a barrier to tumor growth. In the hierarchical cancer model a subset of the cells, the cancer stem cells, divide indefinitely while other cells eventually turn senescent. Here we formulate cancer growth in mathematical terms and obtain predictions for the evolution of senescence. We perform experiments in human melanoma cells which are compatible with the hierarchical model and show that senescence is a reversible process controlled by survivin. We conclude that enhancing senescence is unlikely to provide a useful therapeutic strategy to fight cancer, unless the cancer stem cells are specifically targeted.

Pin1 is a Dlx5 target gene that modulates ΔNp63α protein stability during limb development

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The human Dlx homeobox genes, related to Drosophila distal-less, encode transcription factors that are expressed primarily in embryonic development. Recently, we have reported that the Dlx5 gene is transcriptionally activated by ΔNp63α during mouse limb development. Here, we demonstrate that Dlx5 regulates ΔNp63α protein levels, via proteasome-mediated degradation, activating a negative feed-back loop that requires Dlx5 mediated transcriptional activation of the Pin1 gene. A human Pin1 promoter fragment is activated by overexpression of Dlx5 due to direct binding of Dlx5 to the Pin1 promoter *in vivo* and overexpression of Dlx5 resulted in increased expression of the endogenous Pin1 protein. Pin1 and ΔNp63α interact *in vivo* in a phosphorylation-dependent manner and overexpression of Pin1 resulted in ΔNp63α destabilization. ShRNA-mediated Pin1 silencing resulted in ΔNp63α stabilization and suppresses ΔNp63α degradation induced by Dlx5. Interestingly, ΔNp63α natural mutants associated to SHFM-IV, but not to other p63 related syndromes, were resistant to Dlx5 mediated degradation and a disease-causing Dlx5 mutation abolished its transcriptional activity on the Pin1 promoter fragment as well as activation of the endogenous Pin1 gene. Finally, expression of Pin1 was reduced in p63 and Dlx5/Dlx6 knock-out mouse limbs.
Our data shed new light on the molecular mechanisms regulating the expression of p63 and Dlx5 proteins during limb development and identify Pin1 as a new target gene of the Dlx5 transcription factor.

Neuronal genesis in the striatal parenchyma during progressive degeneration

F. Luzzati[1,2], S. De Marchis[1], R. Parlato[3], S. Gribaudo[1], G. Schütz[3], A. Fasolo[1], P. Peretto[1,2*]
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Acute striatal lesions increase proliferation in the subventricular zone (SVZ) and induce migration of SVZ neuroblasts to the striatum. However, the potential of these cells to replace acutely degenerated neurons is controversial. The possible contribution of parenchymal progenitors to striatal lesion-induced neurogenesis has been poorly explored. Here, we present a detailed investigation of neurogenesis in the striatum of a mouse model showing slow progressive neurodegeneration of striatal neurons, the Creb1(Camkcre4)Crem?/? mutant mice (CBCM). As described in acute models of striatal degeneration, In CBCM mice SVZ neuroblasts migrate to the striatum. These cells follow a specific migratory pathway in which the undergo chain migration through the callosal striatal border and then enter within the striatal parenchyma as individual cells. In addition, a population of clustered neuroblasts showing high turnover rates were observed in the mutant striatum that had not migrated from the SVZ. Clustered neuroblasts might originate within the striatum itself because they are specifically associated with parenchymal proliferating cells showing features of intermediate neuronal progenitors such as clustering, expression of EGF receptor and multiple glial (SOX2, SOX9, BLBP) and neuronal (Dlx, Sp8, and to some extent DCX) markers. Newborn striatal neurons had a short lifespan and did not replace projection neurons nor expressed sets of transcription factors involved in their specification. The differentiation failure of endogenous neuroblasts likely occurred cell autonomously because transplanted wild type embryonic precursors correctly differentiated into striatal projection neurons. Thus, we propose that under progressive degeneration, neither SVZ derived nor intra-striatal generated neurons have the potential to differentiate into striatal projection neurons

p63 regulation of gene expression programs in skin development and disease

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The p53 family member p63 is a tetrameric transcription factor with an essential function in the formation of stratified epithelia both in humans and mice. Heterozygous mutations in p63 are causative of six human malformation syndromes, characterized by various combinations of ectodermal dysplasia, orofacial clefting and limb malformations. In particular Ankyloblepharon-Ectodermal defects-Cleft lip/palate (AEC) syndrome is caused by mutations clustered mostly in the C-terminal domain of the p63 protein, and differs from the other conditions mainly in the severity of the skin phenotype, and the absence of ectrodactyly.
To elucidate the molecular mechanisms underlying this devastating disorder, we recently generated a knock-in mouse model carrying a clinically-relevant mutation in the p63 C-terminal domain. The AEC mouse is characterized by hypoplastic and fragile skin, ectodermal dysplasia and cleft palate, thus mimicking the defects that occur in patients. Epidermal hypoplasia and cleft palate are associated with a transient reduction in epithelial cell proliferation during development due to impaired FGF signaling. Importantly, defects in FGF signaling in AEC syndrome are also associated with reduced epidermal stem cell compartment.
In addition to these defects, AEC mice display epidermal fragility consistent with the skin fragility and erosions observed in patients. Our current studies focus toward the understanding of the molecular mechanism underlying cell adhesion defects, and the exact mechanisms by which mutations in p63 cause impairment of development of several epithelia. Understanding the pathogenetic mechanism(s) at the basis of AEC syndrome will be a prerequisite to identify putative therapeutic strategies.

Dies1: a new player of BMP4 signalling in ESCs

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Embryonic Stem Cells (ESCs) 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. For these characteristics a deep knowledge of the mechanisms that govern ESC fate are fundamental for both basic research and cell replacement therapy. In this context, we have performed a screen based on RNA interference to find molecules regulating ESC fate. Among these proteins, we have identified a transmembrane protein, Dies1, whose knockdown induces loss of differentiation ability in mouse ESCs. This phenotype is accompanied by the downregulation of BMP4 signalling suggesting that Dies1 can participate to BMP4 pathway. We have demonstrated a direct interaction between BMP4 receptor complex and Dies1 by means of FRET microscopy. Moreover, considering that Dies1 suppression resulted in the block of ESC differentiation, we asked whether Dies1 downregulation could be a mechanism that modulates ESC state. Thus we searched for miRNAs regulating Dies1 expression; miRNAs are emerging as a new class of post-transcriptional regulators with a key role in the modulation of gene expression in ESC fate. We have identified a family of miRNAs that are able to regulate the expression of Dies1 and whose overexpression mimics the phenotype induced by Dies1 suppression, indicating that a physiologic regulation of Dies1 levels could have a role in ESC state and/or differentiation.

Human induced pluripotent stem cells (hiPS) as a source of insulin-producing cells

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Background and aims: New sources of insulin secreting cells are strongly required for the cure of type 1 diabetes. Recent success in differentiating human embryonic stem (ES) cells, in combination with the discovery that it is possible to derive human induced pluripotent stem (hiPS) cells from somatic cells, have raised the possibility that a sufficient amount of patient-specific insulin-secreting islet-like cells might be derived from patients cells through cell reprogramming and differentiation.
Methods: We performed the differentiation of hiPS cells in insulin-producing cell optimizing some protocols already established for ES cells. The expression of marker genes of pancreas differentiation was measured through real-time PCR analysis (Taqman) and expressed as fold changes (FC) compared to undifferentiated iPS cells. Proteic expression was confirmed by cytofluorimetric analysis. In vitro differentiated iPS cells were transplanted under the kidney capsule of NOD/SCID mice and the presence of circulating human C-peptide was measured; an immunohistochemical analysis of grafts three months after the transplant was also performed.
Results and conclusions: With our protocol we were able to obtain down-regulation of the pluripotency genes Oct4 and Nanog, up-regulation of the definitive endoderm Sox17 and Foxa2 (39,9±10,1 and 9,3±4,4 FC) and of the pancreatic endoderm genes Pdx1, Ngn3 and Nkx6.1 (34609,2±13151,6, 337,5±164,7 and 7,1±5,4 FC) and the production of Insulin mRNA (132,7±79,4 FC). At the end of the differentiation process 7,7% cells were insulin-positive, as obtained by other groups differentiating ES cells. Preliminary studies in the mouse model show that after glucose stimulus human C-peptide was released in transplanted animals' blood. Differentiated and transplanted iPS cells engraft, survive and do not infiltrate the surrounding tissue. Immunohistochemical analysis revealed the presence of a mixed population, including pancreatic cells.

Direct conversion of mouse and human fibroblasts into functional dopaminergic neurons

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Lineage-specific transcription factors, which drive cellular identity during embryogenesis, have been shown to convert cell fate when expressed ectopically in heterologous cells. Remarkably, developmental fate molecular switchers are sufficient to force neuronal conversion in differentiated somatic cells of other germinal layers. However, at present, it is unclear whether a specific neuronal type can be preferentially induced from direct reprogramming of fibroblasts. Thus, it remains uncertain whether neuronal subtype lineage choices occurring during brain morphogenesis can be reproduced in vitro starting from heterologous cells.
Herein, we screened the key molecular factors governing the dopaminergic (DA) neuronal specification during brain development for their ability to generate functional neurons directly from mouse and human fibroblasts. Remarkably, we found a minimal set of three factors Ascl1, Nurr1 and Lmx1a able to elicit such cellular reprogramming. Molecular and transcriptome studies showed reprogrammed DA neuronal cells to recapitulate gene expression of their brain homolog cells while lacking expression of other monoaminergic neuronal subtype's markers.
Induced DA (iDA) neuronal cells showed spontaneous electrical activity organized in regular spikes consistent with the pacemaker activity featured by brain DA neurons. The three factors were able to elicit DA neuronal conversion in prenatal or adult fibroblasts from healthy donors and Parkinson's disease patients. Generation of iDA neuronal cells from somatic cells might have significant implications in studies of neural development, disease in vitro modeling and cell replacement therapies.
Keywords: Dopaminergic Neurons, Parkinson's disease, Direct cell conversion, Transdifferentiation

Development of pantotenate kinase associate neurodegeneration disease model by direct reprogramming of patients' fibroblasts

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Pantotenate Kinase Associate Neurodegeneration (PKAN) is a recessive, inherited disease characterized by progressive impairment of movement, speech and cognition. This disorder is part of a group of diseases called Neurodegeneration with Brain Iron Accumulation (NBIA). PKAN is caused by mutations of Pantotenate Kinase-2 (Pank-2) gene that encode a mitochondrial protein involved in Coenzyme A synthesis that catalyzes the phosphorylation of vitamin B5. Our previous results on skin fibroblasts from patients and controls showed that the PKAN fibroblasts have an altered oxidative status, in basal condition, and an altered ability to respond to iron supplementation. We are developing a new human neuronal model to further investigate the pathogenetic mechanism and the relationship between iron dyshomeostasis and Pank-2 deficiency. We chose patients carrying two different mutations: a frameshift mutation (F419fsX472) and a substitution (Y190X) that lead premature stop of protein and two controls from ATCC, one from adult subject (X75) and one from neonatal foreskin (neonatal). By lentivirus transduction with combination of three transcriptional factors (Mash1, Nurr1 and Lmx1a) we generated directly dopaminergic neurons from skin PKAN fibroblasts and controls. We characterized the induced dopaminergic neuronal (iDAN) cells with anti β III Tubulin (TuJ1), N-CAM and tyrosine-hydroxylase (TH) staining. The results indicate that the reprogramming efficiency is of about 5% for controls and also for patients. On these human neurons we are evaluating parameters of oxidative status (ROS), iron homeostasis (LIP) and mitochondrial membrane potential variation by using specific fluorescent probes and detecting data at single cell level. Even if the results are preliminary, they suggest that this approach can be utilized for development of human neuronal disease model.

A novel iPSC-based strategy to correct the bleeding phenotype in hemophilia A

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Hemophilia A (HA) is an X-linked bleeding disorder caused by mutations in the coagulation factor VIII (FVIII) gene. Currently, there is no definitive cure. Reprogramming of genetically corrected somatic cells can be used to generate high amount of autologous, disease-free induced Pluripotent Stem Cells (iPSC), which can be then differentiated into progenitor cells relevant for gene and cell therapy applications of HA. Towards this goal, we first generated iPSC from human fibroblasts derived from healthy donors by retroviral transduction with four factors (OCT4, KLF4, SOX2 and c-MYC). These cells were phenotypically similar to human embryonic stem cells (hESC) and expressed specific stem cell markers. iPSC were competent for differentiation into cell types of the three germ layers . Importantly, iPSC differentiated into endothelial cells (EC), a cell type that, when transplanted in HA mice, allows to correct the hemorrhagic phenotype of this model.EC iPSC-derived acquired a typical endothelial-like morphology with increased expression of CD31, vWF and FVIII. Moreover, we recently reprogrammed human and mouse fibroblasts with a Cre-excisable LV expressing OCT4, KLF4 and SOX2. Colonies displayed a hESC-like morphology and stained positive for embryonic stem cell markers. RT-PCR analysis showed activation of the endogenous reprogramming factors in iPSC. Given these results, we reprogrammed HA mouse fibroblasts into iPSC before correction with a LV expressing hBDD-FVIII under control of the PGK promoter and were differentiated in EC. However, in hemophilic patients, to harvest fibroblasts from skin biopsies is risky; for this reason, we used peripheral blood cells as an easy-to-access source of cells and reprogrammed mononuclear cells from donors and hemophilic patients. Overall, these data will be instrumental to assess the engraftment, the proliferation and the levels of FVIII expression from differentiated, gene corrected and reprogramming factor free iPSC to confirm the suitability of this approach for hemophilia gene-cell-therapy.

Distinct roles of NOGO-A and NOGO receptor 1 signaling in the homeostatic regulation of adult Neural Stem Cell activity and in neuroblast migration

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The adult subventricular zone (SVZ) holds GFAP positive Neural Stem Cells (NSCs) able to generate neuroblasts that migrate tangentially within astroglial chains through the rostral migratory stream (RMS) towards the olfactory bulb (OB). We found that plasticity inhibitors Nogo-A and Nogo receptor (NgR1) are differentially expressed in the SVZ-OB system during postnatal development and adulthood. Namely, at both ages, neuroblasts in the SVZ-OB system express Nogo-A, which is instead absent from germinal astroglia and glial tube forming astrocytes. Conversely, migratory neuroblasts never display NgR1 expression, which consistently decorates astrocytes along the ventricles and in the RMS. Indeed, we examined the role of the Nogo-A and NgR1 in the regulation of astrocyte-neuroblast crosstalk in the SVZ-RMS system. By means of pharmacological approaches, we demonstrated that Nogo66/NgR1 interaction actively participates to the inhibition of activation and proliferative activities of adult NSCs in vivo and in vitro. Indeed, neurosphere assay revealed that Nogo-A/NgR1 interactions inhibited neural progenitor stemness, since Nogo66 antagonist NEP1-40 application increases self-renewal and proliferation of SVZ-derived progenitors. Moreover, NEP1-40 application in vivo increases NSC proliferation and a subsequent increase of neuroblast generation. Moreover, we found that Nogo-A delta-20 domain promotes neuroblast migration towards the OB through the activation of the Rho/ROCK pathway without the participation of NgR1. Our findings reveal a novel, unprecedented, function for Nogo-A and NgR1 in the homeostatic regulation of the pace of neurogenesis in the adult SVZ and in the migration of neuroblasts along the RMS.

Generation of new tools and iPS cell-based in vitro model systems to study Amyotrophic Lateral Sclerosis (ALS)

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Amyotrophic Lateral Sclerosis (ALS) is one of the most severe neurodegenerative disorders, due to the loss of upper and lower motor neurons. A subset of familial ALS cases is linked to mutations in the FUS/TLS and TARDP genes, both encoding for putative regulators of microRNA (miRNA) biogenesis. In the nervous system, miRNAs fine-tune neuronal gene expression in a spatially and temporally restricted manner and deregulation of specific miRNAs correlates with the initiation and progression of several neurological disorders. Our general aim is to study the possible involvement of miRNAs in ALS pathogenesis.
One of the major problems with the molecular analysis of the ALS-associated mutations is the lack of suitable cellular model systems. In order to circumvent this issue, our approach consists in the generation of pluripotent stem (iPS) cell lines from fibroblasts of ALS patients, which can be differentiated into genetically matched motor neurons. A collection of ALS-iPS covering several FUS/TLS and TARDP mutations is now available in our lab and can be exploited for the analysis of possible alteration of RNA metabolism in ALS motor neurons. We will also describe a new transposon-based vector for transgenesis of human cells, including iPS cells. In particular, possible applications of our vector in the study of ALS include: the inducible ectopic expression of neuronal miRNAs or mutated ALS genes; the analysis of mislocalization of ALS mutated proteins in live cells; the isolation of mature motor neurons during differentiation.

The role of ArhGAP15, a negative regulator of the small-GTPase Rac1, in neuronal migration and neuritogenesis during cortical development

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Forebrain development requires the coordination of neurogenesis, migration, differentiation and neuritogenesis-synaptogenesis, processes regulated by a complex network of molecular players. Rho-GTPases are a class of transducing enzymes with key roles in the cytoscheletal organization in response to signals; Rac1 is a GTPase essential for neurite elongation and synaptogenesis, via regulation of dynamic actin. Rho GTPases are generally activated by GEFs and inactivated by GAPs. Recently we found the Rac1-GAP ArhGAP15 (AG15) expressed in olfactory and cortical interneurons. We have generated AG15 knock-out (KO) mice; in these we notice an increased Rac1 activity, confirming the loss of AG15-dependent inhibition. We examined primary cultures from the cortex of wild-type and AG15 KO embryos; we observe a significant reduction in neurite length and branching of interneurons, in the absence of AG15. On this basis, we postulate that misregulation of Rac1 and of cytoscheletal dynamics leads to abnormal early neuritogenesis and possibly affects migration. We therefore examined the distribution of interneurons in the cortex: while no significant difference was seen in the adult cortex, in the P0 AG15 KO mice we find an abnormal stratification, suggestive of migration impairments. However, tangential migration of immature interneurons in the embryonic cortex is not apparently affected. To visualize neuronal migration in prenatal stages in vivo, we crossed AG15 mutant animals with GAD1-GFP transgenics, and performed time-lapse microscopy of the cortex. Based on our results, we propose that in the absence of AG15 (i.e. hyper-active Rac1), the switch from tangential to radial migration is impaired, while tangential migration per se is normal. Interestingly, this migratory switch is also impaired in AG15 mutant macrophages and neurotrophils. With this hypothesis, we will further examine the role of AG15 in neuronal migration, focusing on actin dynamics.

Establishing a fast method for the generation of functional dopaminergic neurons from human iPS cells

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Current protocols for in vitro differentiation of human induced pluripotent stem cells (hiPSCs) for generating dopaminergic (DA) neurons are time expensive requiring a series of subsequent steps. In order to speed up the overall process of neuronal generation devising a straightforward approach, we have established a fast protocol by means of lentiviral overexpression in hiPSCs of DA-lineage specific transcription factors. With this method, we were able to generate mature and functional dopaminergic neurons within only 20 days and skip the steps of induction and selection of the rosette-neural precursors. Strikingly, this neuronal conversion process resulted very proficient with an overall efficiency of 64%. Infected iPS-derived DA neurons expressed all the critical molecular markers of the DA molecular machinery and exhibited sophisticated functional features including spontaneous electrical activity, synaptic currents and dopamine release.
This one-step protocol holds important implications for in vitro disease modeling and is particularly amenable for exploitation in high-throughput screening protocols.

Role of p130Cas in the control of epithelial cell commitment and differentiation in the mammary gland

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Like most adult tissues, the mammary epithelium has a hierarchical organization: stem cells via a series of progenitors give rise to two major cell types, the basal and the luminal cells, which include ductal and alveolar cells. However, signaling pathways that specify mammary lineages are not well known. We previously reported that the adaptor protein p130Cas is upregulated in breast cancers and that its overexpression promotes mammary tumorigenesis. Here we describe how high levels of p130Cas affect cell fate decisions and normal homeostasis in the mammary epithelium. By a deep analysis of MMTV-LTR-p130Cas transgenic mice, which overexpress p130Cas in the mammary gland, we found that MMTV promoter-driven p130Cas overexpression is mainly addressed to the mammary progenitors and profoundly alters the cell composition of the mammary epithelium. Indeed, MMTV-p130Cas mammary cells display a strong upregulation of genes peculiar of basal cells. Moreover, progenitors from MMTV-p130Cas glands preferentially differentiate into basal rather than luminal cells in vitro. In addition, during pregnancy transgenic glands show an aberrant alveolar development as well as an accumulation of immature alveolar progenitors and reduced levels of milk proteins. It has been recently reported that mammary progenitor cells, the major target for the MMTV-LTR in our mouse model, highly express the tyrosine kinase receptor c-kit. Interestingly mouse mammary epithelial cells (MMECs) from MMTV-p130Cas animals exhibit hyperactivation of c-kit in the presence and even in the absence of its ligand. Notably, wt MMECs transduced to express a constitutively active c-kit result biased towards a basal differentiation route and refractory to mature into alveolar cells, thus clearly mimicking p130Cas overxpressing cells. Together these data shed light on a novel function for p130Cas and c-kit, whereby p130Cas seems to act in the control of mammary cell differentiation.

FVIII expression and secretion in bone marrow cells capable of correcting bleeding in hemophilia A mice

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Identification of cells capable of synthesizing and releasing factor VIII (FVIII) is critical for developing therapeutic approaches in hemophilia A (HA). Endothelial cells(EC), particularly liver sinusoidal endothelial cells (LSEC), express FVIII most in the body. However, recent studies of bone marrow (BM) transplantation suggested additional cell types could synthesize and release FVIII, and also correct bleeding in HA mice. Therefore, to establish the ability of circulating blood cells in expressing FVIII, we analyzed several murine and human hematopoietic cell types. First, we generated polyclonal antibody against recombinant human FVIII. The specificity of this FVIII antibody was established by western blotting, staining of lentivirally transduced fibroblasts from HA mice expressing B domain-deleted FVIII. Second, we found by immunostaining that FVIII was present in hematopoietic cells isolated from peripheral blood, BM and human cord blood (hCB). The identity of these cell types was verified by costaining for FVIII and cell type-specific markers for monocytes, dendritic cells and megakaryocytes. Moreover, FVIII expression in these cell types was verified by RT-PCR and western blot. Third, antibody staining confirmed FVIII expression in normal human liver, including LSEC, Kupffer cells (KC), and hepatocytes. Also, we observed FVIII expression in mononuclear or EC in other organs, e.g., spleen, lungs and kidneys. Finally, transplantation studies were performed in HA mice with either wild-type mouse KC or CD11b+ monocytes from hCB. Tissue analysis showed transplanted cells entered the mouse liver and survived for at least 1 w. Tail clip challenge in HA mice showed correction of bleeding in 9 of 14 mice (64%) after KC, and 9 of 11 mice (82%) after cells from hCB in NOD/SCID HA mice. We confirmed plasma FVIII activity in several surviving HA mice. Conclusions: Besides EC, FVIII was expressed in circulating blood cells, offering further opportunities for understanding mechanisms in FVIII synthesis and replenishment.

ABSTRACTS

Poster Presentations

in alphabetical order
(presenting authors are shown underlined)

A genome-wide screening to identify microRNAs regulating stem cells self-renewal

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MicroRNAs are short (about 18-22 nucleotide long) RNA molecules recently emerged as pivotal regulators of gene expression at post-transcriptional level in most organisms. MicroRNAs participate at the regulation of almost every cellular process and changes in their expression are frequently associated with human pathologies. Lately, microRNAs have been also described as players in stem cell fate by acting on self-renewal and differentiation process. Stem cells are typically characterized by the ability to give rise to different specialized cell types (pluripotency) and to divide mitotically to maintain stem cell compartment (self-renewal). Usually, stem cells (SC) division occurs asymmetrically, thus generating one daughter cell that retains stem cell properties and another daughter cell (progenitor) that is committed to differentiation. However, in some cases stem cells division occurs symmetrically, thus leading to the expansion of stem cell pool.
A number of evidence suggests many cancers (and breast cancer in particular) are organized as abnormal tissues containing a subset of cells with SC-like properties (cancer SCs or tumor-initiating cells). CSC are able to sustain tumor growth, resist to chemotherapy and ultimately drive metastasis. Indeed, our lab has recently reported that the molecular and clinical heterogeneity of breast cancers is a function of their cancer SC content, underlining a different self-renewal capacity of CSCs.
Based on this background, the aim of this project is to explore the role of microRNAs as regulators of self–renewal for both breast SCs and CSCs through a phenotype competition assay based on mammospheres formation.
By exploiting a genome-wide approach we will identify those microRNAs which either favor or inhibit self-renewal of breast SCs and CSCs.
The results of this study will be of fundamental importance in highlighting pathways and mechanisms which control stem cell biology under physiological and pathological conditions and provide, at the same time, a novel set of molecules with diagnostic and therapeutic potential.

Exposure of mesenchimal stem cells to estrogen and growth hormone induces osteogenic commitment even in an adipogenic culture environment

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Bone loss in aging is multifactorial and hormone decline is a major contributing factor. Senescence of bone is accompanied by an increase in adipogenesis to the disadvantage of osteoblastogenesis. In women, aging is associated to a sharp decline in two of the main anabolic hormones, estradiol (E2) and growth hormone (GH). It is known that E2 is able to favour mesenchimal stem cell (MSC) osteogenic commitment (Zhao et al, 2011), and that an impairment in GH/IGF1 axis is related to osteopenia (Perrini et al, 2010). Therefore it is likely that the simultaneous fall of these two hormones could be involved in the reduced osteoblast formation in aging.
In order to evaluate the effect of GH (5ng/ml) or E2 (10-8M) or the combination on MSCs commitment, cells were cultured with either the basal osteogenic or adipogenic differentiation medium for a period of 14 or 28 days. MSCs are able to respond to GH through the activation of JAK/STAT pathway; E2 alone doesn't activate this pathway. MSCs cultured in adipo-medium and treated with GH up-regulate the expression of Runx2, an osteo-differentiating transcription factor, both at 14 and 28 days compared to the controls. E2 treatment 60 min before GH amplifies GH effect on Runx2 at day 14, but not at 28; whereas E2 per se down-regulate it at both 14 and 28 days. GH and E2 alone decrease adiponectin gene expression after 28 days and the association of the two hormones does not potentiate the effect. MSCs cultured in basal osteo-medium in the presence of both GH and E2 increase Runx2 expression at 14 days and the two hormones either alone or in combination up-regulate osteoprotegerin expression.
From the results it emerges that E2 in association with GH favours MSC commitment towards osteoblastogenesis also when cells are cultivated in an adipogenic environment.

The neurotrophin receptor p75 identifies cell line subpopulations displaying stem hallmarks and enhanced invasive growth ability *in vivo*

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The low-affinity neurotrophin receptor (p75-LNGFR) is a putative marker of neuroectodermal stem/progenitor cells. We found that p75 is abundantly expressed in subpopulations of cell lines from tumors of neuroectodermal origin (glioblastoma, neuroblastoma and melanoma). In the case of glioblastoma and neuroblastoma, p75 expression was found to colocalize with stem markers and was increased in neurospheres formed by cell lines kept in culture conditions selecting for stem cells. Sorting for p75 expression allowed to discriminate cell subpopulations with different phenotypes, in some cases recognizable at morphological level (glioblastoma SNB75). Unlike p75-, p75+ cells could form neuro- or melanospheres, and, in standard culture conditions, reconstituted a mixed population including both p75+ and p75- cells. Moreover, the p75+ subpopulation was slower cycling and more resistant to apoptosis than the p75- subpopulation. After orthotopic transplantation in immunocompromised mice, the p75+ subpopulation sorted from neuroblastoma LAN5 displayed significantly enhanced tumorigenic ability as compared with the p75- subpopulation, resulting in reduced mouse survival, increased tumor volume and metastatic dissemination. These data indicate that, in cell lines of neuroectodermal origin, the p75+ subpopulation holds stem-like properties in vitro and increased tumorigenicity in vivo.

Protracted neuro/gliogenesis in the CNS parenchyma: effect of age, species, lifespan

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Adult neurogenesis in mammals is mainly detectable in highly restricted brain sites. Persistent neurogenesis depends on neural stem cells residing in niches which are remnants of the embryonic germinal layers. Local progenitors which retain some proliferative capacity also exist in the mature brain parenchyma. In rodents, usually they do not support an 'actual' neurogenesis, but rather a potentiality which does not manifest spontaneously in vivo. In contrast with such a view, genesis of neuronal and glial cells from local progenitors does occur in the peripuberal and adult rabbit cerebellum. This process, which is independent from persisting germinal layers, involves different cell populations: Pax2+ interneurons, Bergmann glia, and multipolar, Map5+/Olig2+ cells (Ponti et al., 2008, Plos One). The genesis of Bergmann glia and Pax2+ interneurons persists mainly during postpuberal stages, falling at very low levels at 1 and 3 years of age. In lagomorphs, which have a longer lifespan with respect to rodents, these processes can be linked to a protracted growth in young animals, whereas in rodents, characterized by fast growth and short lifespan, they sharply cease before puberty. The Map5+ cell population also appears reduced in size with age, although to a lesser extent. Interestingly, Map5 (microtubule-associated protein 1B) is expressed both in neurons and glial-like cells. The Map5+ multipolar cells are still detectable at later ages. We analysed 7 mammalian species, including humans. These cells co-express the GPR17 receptor and are postmitotic (the Map5 staining appearing two weeks after their birth), likely being part of the Ng2+ cell lineage at a pre-myelinating stage.
We conclude that mammalian parenchymal cell genesis, can be a protracted rather than a persistent process, which is differently regulated by age, species and lifespan.

Rat bone regeneration induced by the engraftment of human amniotic fluid stem cells (AFSC) unselected for c-Kit: a pilot study

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Amniotic fluid contains pluripotent progenitor cells that can differentiate *in vitro* into cell types from all three germ layers and that do not induce teratomas *in vivo*(1). Although various stem cell sources have been investigated for bone repair, few comparative reports exist, and cellular distribution and post-implantation viability remain key issues(2). The present study was done with human AFSC expanded *in vitro* up to the 4th passage in proliferation medium and pre-labelled with a fluorescent cell permeable dye (PKH26) in order to track their *in vivo* distribution. For *in vivo* experiments male rats (48-50 week-old; 410-480 g) were injured at the femoral diaphysis in order to produce a 5-7 mm diameter full thickness bone gap. The femoral defect was left untreated (control rats) or filled with HA (natural nanocrystalline carbonated hydroxyapatite-Orthoss®) scaffold alone or loaded with PKH26-labelled AFSC. Three weeks after implantation all animals were sacrificed and femoral specimens were explanted, fixed with 10% buffered formalin, decalcified and processed for conventional paraffin embedding and staining for light microscopy. Both gross anatomy and histological observations revealed a major bone regenerative response in rat specimens treated with HA scaffold alone or supplemented with AFSC. In particular, samples injected with HA plus AFSC displayed the formation of periosteal woven bone and an increased presence of blood vessels in the bone marrow, with still fluorescent AFSC in close proximity. Taken together, these preliminary observations provide evidence that amniotic fluid represents a new and very promising source of engraftable stem cells for treating human orthopaedic disorders (such as non-unions and bone defects) as an alternative therapeutic strategy to bone grafting operations.

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Patient-specific induced pluripotent stem cells as a human-derived model to study metachromatic leukodystrophy

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Metachromatic leukodystrophy (MLD) is a neurodegenerative pediatric Lysosomal Storage Disorder (LSD) caused by genetic defects in the activity of arylsufatase A (ARSA), a key enzyme in the catabolism of myelin-enriched sphingolipids. While primary genetic and biochemical defects are described in LSD, the paucity of currently available human-derived study models hampers accurate exploration of downstream events in relevant target cells, like neurons and oligodendrocytes. To overcome this limitation we propose to establish patient-specific induced pluripotent stem cells (iPSCs) by somatic cellular reprogramming and to generate iPSC-derived neurons and glia. For the gene replacement strategy, we transduced fibroblasts derived from MLD patients with a bidirectional lentiviral vector (bdLV.GFP.hARSA-HA) ensuring a supraphysiological ARSA activity. For the reprogramming process, we transduced WT, MLD and gene-corrected MLD fibroblasts with a monocistronic LV encoding the human OCT4, KLF4 and SOX2 genes. We have expanded the iPSC clones to generate iPSC lines, which have been characterized for the expression of pluripotency markers and for the ability to induce teratoma formation upon sub-cutaneous injection in immuno-deficient mice. Preliminary data show that all iPSC lines are able to differentiate in immature neurons and astrocytes. We are optimizing a protocol to obtain iPSC-derived neural stem/progenitor cells to be used as a renewable source of patient-derived neural cells that will be further differentiated in cultures enriched in oligodendrocytes and mature neurons. On these patient-specific iPSC-derived neural cultures we will assess changes in survival, growth, biochemical, molecular and epigenetic signature, trying to model the degenerative mechanisms, to test the efficacy of gene correction in reverting the disease phenotype and, ultimately, to test safety and efficacy of iPSC-derived neural cells in MLD murine models.

Development of a Cancer Stem Cell based *in vitro* model to be used for pharmacological assays in oncology

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Cancer Stem Cells (CSCs) are a rare population of undifferentiated tumorigenic cells responsible for tumor development, maintenance and spreading. These cells represent the only tumor population able to proliferate indefinitely in vitro and to reproduce tumor xenografts identical to the original tumor (both at morphological and biomarkers level) when transplanted in vivo into immunodeficient mice (Singh et al, 2004; O'Brien et al, 2007; Ricci-Vitiani et al, 2007).
Cancer drug discovery process is largely based on several in vitro assays, usually set up on cancer cell lines in order to test the potential effect of molecules during preclinical R&D. These tests have been demonstrated to be poorly predictive of the efficacy of clinical trials. Similarly, since the standard primary tumor cultures are unable to allow the growth of cancer-initiating cells endowed with self–renew capacity, also tests on primary tumor cells could be poorly indicative of therapeutic efficacy.
In order to improve identification of powerful therapeutics in oncology, we have developed and validated a Cancer Stem Cell based in vitro model. IOM Ricerca srl offers a screening service on in vitro models of cancer stem cells (CSCs), for evaluation of the pharmacological activity of new molecules, 'lead compounds', innovative formulations of existing molecules or therapeutic combinations. Specifically, in vitro models of CSCs are set up from a variety of tumors, including glioblastoma, melanoma, lung, thyroid, breast and colon cancer.
IOM Ricerca srl business model also includes Collaborative Research on specific Projects using CSCs.

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Modulation of blood brain barrier permeability for a better access of anti-cancer drugs to brain tumors: the CCM/β-catenin crosstalk

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Primary brain malignancies and brain metastasis are more resistant to chemotherapies compared to other types of tumors. This is due to the very specialized phenotype of the brain vessels, the so-called blood brain barrier (BBB). BBB limits the passage of anti-cancer drugs from the blood to the brain. Therefore, it would be therapeutically useful to develop systems to modulate BBB permeability. To this aim it is important to define the molecular mechanisms that regulate the establishment and maintenance of BBB properties.Data from our laboratories, suggest a key role of the Wnt/β-catenin signaling pathways in the induction, regulation and maintenance of the BBB characteristics during embryonic and post-natal development. In endothelial cells, Wnt signaling induces barrier differentiation by increasing the stabilization and the transcriptional activity of β-catenin. On the contrary, inactivation of β-catenin causes significant downregulation of junctional proteins, and consequent BBB breakdown.Besides β-catenin, other three proteins, CCM1, CCM2 and CCM3, expressed by brain endothelial cells, are emerging as key modulators of the organization and function of the BBB. Indeed, mutations occurring in any of the genes encoding these proteins, leads to Cerebral Cavernous Malformation (CCM), a pathology characterized by brain vascular malformations.
The endothelium in the lesions presents very few tight junctions and gaps are observed between endothelial cells. In addition, the vascular basal lamina is disorganized and the astrocytes do not take contact with the endothelial wall. The structural alterations of the BBB observed in CCM lesions are associated with severe clinical manifestations, such as cerebral haemorrhages and stroke. Additional data point to a possible link between the β-catenin pathway and the functions of CCM proteins in the regulation of BBB stability. These data open new therapeutic opportunities for this so far incurable disease.

Morphological, molecular and functional differences of adult bone marrow- and adipose-derived stem cells isolated from rats of different ages

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The transplantation of primary Schwann cells (SC) has been shown to improve nerve regeneration, however clinical use of autologous SC for the treatment of nerve injuries is of limited use due to difficulty in obtaining clinically useful numbers. In vitro, adult mesenchymal stem cells have self-renewal and multiple differentiation potentials and play an important role in regenerative medicine. However, their use may be limited by senescence or by the age of the donor, which may lead to changes in stem cell properties and functionality. Hence, it was of interest to determine the possible morphological, molecular and functional differences of bone marrow-derived (MSC) and adipose-derived (ASC) stem cells isolated from animals of different ages. Immunocytochemistry, RT-PCR, proliferation assays, Western blotting and transmission electron microscopy techniques were used to investigate the expression of senescence markers. The proliferation rates, expression of senescence markers (p38 and p53) and the stimulation of neurite outgrowth from DRG neurons by stem cells isolated from neonatal, young or old rats were very similar. Undifferentiated and differentiated ASC and MSC from animals of all ages expressed Notch-2 at similar level. Also, following co-culture with neuronal cells, dMSC and dASC elicited neurite outgrowth. TEM analysis confirmed the mitochondria staining in which ultrastructural changes in mitochondria are initial sign of senescence. Overall, the results of this study showed that aged MSC and ASC differentiated into SC-like still retain the potential to support axon regeneration although the aged cells characterized in this study expressed markers typical of senescence.

Development of GABAergic interneuron progenitors in the postnatal cerebellar white matter

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The cerebellar prospective white matter (PWM) is a secondary germinal site, active during late embryonic life and postnatal development. Its cellular composition is complex, including cells with stem-like properties, dividing progenitors, and postmitotic elements at different maturation stages. PWM cells give rise to GABAergic interneurons, astrocytes and oligodendrocytes, but the relationships linking these lineages are largely unclear. Our interest is focused on the development of GABergic interneurons: a distinctive marker of this lineage is the transient expression of the transcription factor Pax-2 that is upregulated during the last cell division within the PWM. However, little is known about PWM-progenitor cells before the onset of Pax-2 expression. To clarify these aspects, we first evaluated environmental factors that could be involved during the development of interneuron progenitors in the PWM. We found that Sonic Hedgehog (Shh) can influence the amplification and differentiation of Pax-2-positive pools. Furthermore, to investigate the mechanisms of GABAergic interneurons specification and differentiation, we examined their lineage relationships with the other PWM-derived phenotypes. A genetic fate mapping study of GlastCreERT2 mice showed that cerebellar GABAergic interneurons derive from proliferating Glast-positive precursors that could be shared with astrocytes. The same conclusions were supported by injection of lentiviral vectors that preferentially infects astroglial cells. Infected cells in embryonic or postnatal cerebellar generated a mixed population of interneurons and astrocytes. In conclusion, our results indicate that progenitors with astroglial features are present in the postnatal cerebellar PWM. These progenitors are able to differentiate both into interneurons and into astrocytes, indicating a possible relation between these two lineages.

Thyrospheres with stem-like properties derived from the papillary carcinoma B-CPAP cell line

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Cancer Stem Cells (CSCs) have been detected in several kinds of tumors and in cancer cell lines. To the best of our knowledge, no studies have been reported on CSCs from papillary thyroid carcinoma (PTC)-derived cell lines. We identified and characterized cancer stem-like cells from the PTC-derived B-CPAP cell line. The SV40 transfected follicular epithelial cell line NThy-ori 3-1 has been used as control. The stemness profile was evaluated by functional assays and RT-PCR. Thyrospheres from B-CPAP cells were able to propagate up to 10 generations, with sphere forming efficiency and self renewal increasing exponentially up the 8th. By contrast, the control cells were able to propagate up to the 4th generation, progressively decreasing sphere forming efficiency and self renewal.
B-CPAP thyrosferes express Oct 4, Nanog, and ABCG2 stem cell markers, PAX8, TTF1, and Tg thyroid differentiation markers, and p63 tumor marker. Control thyrospheres were positive for stem cell markers and PAX8, but they did not express p63 and differentiation markers.
Stem-like cells from tumoral and control thyrospheres, were isolated by flow cytometry cell sorting assay. The ability to retain the lipophilic fluorescent dye PKH26, as a consequence of their quiescent nature, has been used to selected the brightest (PKH26 High) and the dimnest (PKH26 Low) cell populations. PKH26 High only, which constitutes the putative stem cells, retains the ability to form secondary spheres and shows increased expression of stem cell markers compared to PKH26 Low, which represents the proliferating and differentiated cells. As expected, differentiated markers were more expressed in PKH26 Low. Taken together, our data show that B-CPAP cell line contains cells with stem-like features and properties.

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Key words: cancer stem cells, papillary thyroid carcinoma, thyrospheres

The endocannabinoid system in immortalized neural progenitors (ST14A cells): a role for CB2 receptors

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According to recent views, endocannabinoids may constitute a new group of lipid signalling cues involved in the regulation of neurogenesis, in addition to actively intervening in different physiological brain contexts. The presence of a functional endocannabinoid system (eCBs) in neural progenitor (NP) cells may indicate a role of these molecules in important processes such as cell proliferation and differentiation. We have investigated this occurrence in the ST14A immortalised neural progenitors cell line, derived from embryonic day 14 rat striatum primordia.
We found by RT-PCR, ICC and WB that ST14A cells express various components of the eCBs, including synthetic and degradative enzymes, several receptors among which TRPV1, cannabinoid receptor1 (CB1) and 2 (CB2). In particular, CB2 was more abundantly expressed than CB1 under both proliferating and differentiating conditions. By incubating ST14A cells for 24 hrs with serial concentrations of 2-arachidonoylglycerol (2-AG), an increase in the number of cells was found at 5?M. A similar increase was found by incubating the cells with a CB2 specific agonist (300nM JWH133). Such effect was reverted by co-incubation with a CB2 specific antagonist (300 nM AM630), which had no effect when administered alone. A proliferation assay based on BrdU incorporation confirmed the above results. Changes in cell morphology and in the staining pattern of cytoskeletal components, such as the intermediate filament nestin, were observed in the various experimental conditions.
Our data demonstrate the expression of a full eCBs and suggest its modulatory role in proliferating ST14A neural progenitors, setting the basis for future investigation of the intracellular mechanisms involved and possible crosstalks with other signaling systems. Differential modulations related to specific cell cycle and maturation stages are also important issues to be clarified.

ΔNp63α acetylation is impaired in natural p63 mutants

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The p63 transcription factor, homolog to the p53 tumor suppressor, plays a crucial role in epidermal and limb development. Dominant mutations in the *p63* gene give rise to several human congenital sindromes like the Ectodactyl-Ectodermal displasia-Clefting (ECC), Ankyloblepharon-Ectodermal displasia (AEC), Limb-Mammary Syndrome (LMS) and Split-Hand/Split-Footh Malformations (SHFM) type IV syndrome. Little is known on the post-translational modifications controlling p63 functions. On the other hand, it's well known that p53 transcriptional activation is dependent on lysine acetylation driven by the p300 acetyl-transferase. Recently, a new lysine acetylated by p300 was identyfied in p53 (K164). This lysine is well conserved in p63 and p73 and it corresponds to K193 in the p63 protein. Natural mutations of this lysine to glutamic acid (K193E) are associated to the human SHFM-IV syndrome.
We have evidences that p300 acetylates, in vitro, a p63 peptide centered on lysine 193. Furthermore, p300 interacts with p63 in human cell lines and p300 overexpression increases p63 half-life whereas shRNA mediated p300 silencing reduces p63 stability; interestingly, the p63K193E mutant is insensible to p300 overexpression. In addition, p300 enhances p63 transactivation potential on the *Dlx5* and *Dlx6* promoters, while the activity of the K193E mutant is not influenced by p300 co-expression; interestingly, the K193E mutation seems to be a promoter specific mutation, since it has reduced transactivation potential on development related promoters (*Dlx5* and *Dlx6*) while it has a normal transactivation on a cell-cycle regulated promoter (*p57kip2*).
Taken together our results suggest that p300 acetylates p63 on K193: the importance of this post-translational modification is underlined by the fact that this lysine is found mutated in SHFM-IV patients.

Effects of electric stimulation on human progenitor/stem cells from different sources

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Introduction and Aims. Electric stimulation (ES) can induce the commitment of stem cells towards differentiation, for example cardiomyogenic and, nervous. The effects of mono- and bi-phasic electric stimulation on myogenic commitment are still poorly understood and thus the aim of this work was to investigate these effects on a mouse stem cell line (m17.ASC) and on adult stem cells isolated from human heart and human adipose tissue.
Methods. *Device*: the bioreactor system consists of a chassis with an electrical wiring system and is equipped with housings for multiple culture PDMS chambers, in which stainless steel electrodes are embedded. Electrical stimulation is driven by graphical-interface software..
Biological experiments: the m17.ASC cell line and human adult stem cells were seeded on glass slides and 24 hours later the EFs (square mono-phasic 2ms, 1Hz, 5 and 8V amplitude or bi-phasic 2ms, 1 Hz, ?2.5 and 4 V amplitude) were applied for different time points (from 3 h to 6 d). Cell proliferation and commitment were evaluated by crystal violet staining and on the basis of the expression of the early cardiac markers Connexin 43 (Cx-43) and Gata-4 by IF respectively.
Results. m17.ASCs upon both electrical stimulation protocols showed proliferation rates similar to those of not-stimulated cells. For human cells proliferation decrease upon bi-phasic ES if compared to not stimulated cells. After 3 days of biphasic stimulation m17.ASCs up-regulated the expression of Cx-43. Similar results were observed for both human cell types at 3 and 8 hours. In the case of human cells bi-phasic stimulation induced also the up-regulation of GATA-4 transcription factor.
Conclusions. These results suggest that the bi-phasic EF can be an appropriate signal to induce myogenic commitment in mesenchymal stem cells. Experiments which combine this stimulus with stiffness and topography of the culture substrate are in progress.

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Design and layout

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