UNIVERSITÀ DEGLI STUDI DI MILANO

FACOLTÀ DI MEDICINA E CHIRURGIA

SCUOLA DI DOTTORATO IN SCIENZE BIOMEDICHE CLINICHE E SPERIMENTALI

DIPARTIMENTO DI BIOTECNOLOGIE MEDICHE E MEDICINA TRASLAZIONALE
(BIOMETRA)

DOTTORATO DI RICERCA IN

PATOLOGIA E NEUROPATOLOGIA SPERIMENTALI

(XXVI CICLO)

TESI DI DOTTORATO DI RICERCA

"CORRECTION OF AN X-LINKED GENETIC DEFECT BY MICROCELL-MEDIATED CHROMOSOME TRANSFER"

(MED 03/04/05)

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Anno accademico 2012/2013

Ai miei cuori.

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1. INTRODUCTION

1.1. STEM CELLS

Stem cells are a very important resource in terms of cell renewal. They are considered as the organizational units responsible for the development and regeneration of tissues and organs, and are broadly defined as clonogenic cells, because they can both proliferate independently as well as to differentiate into various cell populations. In fact, *in vivo* they maintain themselves thanks to an asymmetric cell division: after the cell duplication, a daughter cell becomes "committed" and moving towards a specific cell type, while the other one remains undifferentiated, to maintain a constant reserve of potentially usable cells (He et al., 2009; Sylvester and Longaker, 2004). Stem cells are generally classified into four different types, according to their degree of "potency", understood as the ability to differentiate and specialize in different cellular types: totipotent, pluripotent, multipotent and unipotent cells (**Fig.1**).

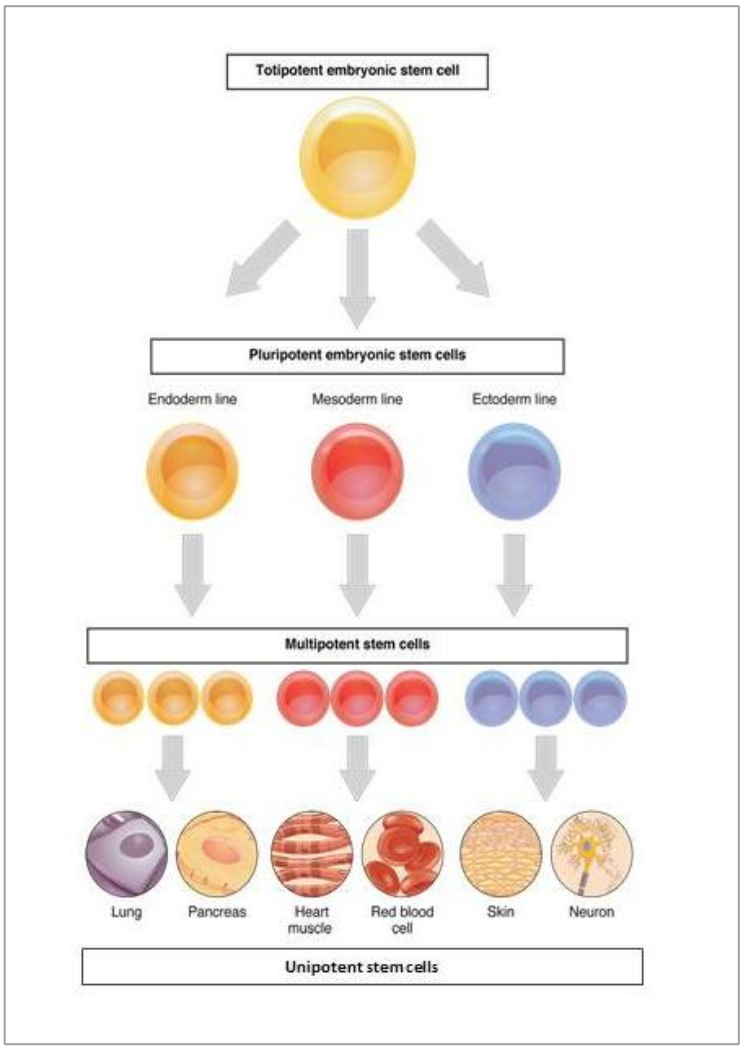

Fig 1 - Different grade of "potency" of stem cells. Starting from the morula and blastocyst stage, in which stem cells are respectively totipotent and pluripotent, so potentially able to generate in the first case all individual tissues including extra embryonic (placenta) tissue and the second case any kind of cellular types, the cells progressively specialize and become committed, able to generate only a few cell types.

Totipotent cells have the potential to give rise to a whole organism, including extra-embryonic (placenta) tissue, so they are totally "un-committed", and represent the earliest stage of un-differentiated cells. Pluripotent cells are able to differentiate into all cells that constitute an individual but they don't give rise to extra-embryonic tissue; being able only to generate all three germ layers cells. Multipotent stem cells have a much less potency because they have the ability to differentiate only in certain cell types (i.e. the hematopoietic stem

cells), as these cells are already genetically directed towards a cell type. Finally, the unipotent cells are able to differentiate only in a specific differentiated class of cells. The latter represent the lowest potency level within the scenery of stem cells.

Stem cells can also be divided in embryonic, fetal and adult stem cells, with respect to their potency and their developmental stage derivation (Czyz et al., 2003). Embryonic stem cells are found in the embryo in a very early stage of development, and they are considered pluripotent stem cells. Fetal stem cells are derived from an embryo at the fetal stage, from the biological point of view there is not yet a definitive characterization, but available studies showed that they have intermediate characteristics between embryonic and adult. The adult stem cells are instead partially specialized cells, responsible for the regeneration of damaged tissues, as well as involved in the normal homeostasis of the tissue. The adult stem cells constitute a great therapeutic hope in man, since they could be drawn out from an individual, corrected and used in the patient for cell therapy, without incurring in ethical problems (Ellison et al., 2013). In recent years research using stem cells is moving on induced pluripotent stem cells (IPSc), adult cells already differentiated brought at the pluripotent stage, by inducing a "forced" expression of specific genes. One of the most applications of stem cells is in the care and treatment of a wide variety of movement disorders including Parkinson's disease (PD) or and Huntington's disease (Schwarz and Schwarz, 2010).

Another important line of research that is increasingly being developed in recent years, is about a class of stem cells defined as mesenchymal stem cells (MSC), that are found in different points of the body and are able to generate bone tissue, cartilage or adipose tissues (Dimarino et al., 2013; Stewart and Stewart, 2013). Cultures of MSCs have already been established in the early 1960s when fibroblastoid cells were discussed as supportive stromal cells within the hematopoietic bone marrow niche. Such cells, with a morphology similar to fibroblasts, with a low amount of cytoplasm, mitochondria, and a little

developed Golgi apparatus, are usually believed to be multi- or oligo-potent stem cells that can be expanded *ex vivo* and have the potential for differentiation into at least three cell types: osteocytes, chondrocytes, adipocytes (Minguell et al., 2000; Minguell et al., 2001).

Despite the ethical problems concerning the use of human embryonic stem cells, their ability to generate any type of tissue present in the body make them a fundamental tool for basic research. Many research groups have been and are moving to the field of basic research using mouse stem cell to understand the mechanisms that underlie many biological and pathological processes that affect humans, being these cells an excellent reference model.

1.1.1. MOUSE EMBRYONIC STEM CELLS

"Pluripotential embryonic cells appear to give rise to both rapidly differentiating cells and others which like themselves, remain undifferentiated" (Stevens and Little, 1954).

These words were used by Stevens and Little in 1954 while describing ovarian teratomas; several years later this description became the best definition of embryonic stem cell. The earliest pluripotent stem cells were isolated from teratocarcinoma, complex malignant tumors composed by different types of cells resulting from three germ layers and undifferentiated cells, and were called embryonic carcinoma (EC) cells (Martin and Evans, 1975). The limit of using these cells as a model is their instability in culture, because of their tendency *in vitro* to aneuploidy (Martin, 1981). This causes the limited use of these cells for genetic engineering because they rarely colonize the germ line.

In 1981, two reports for the first time showed that was possible to isolate from the inner cell mass (ICM) of a mouse embryo at the blastocyst stage cells that were able to grow *in vitro,* and to establish permanent mouse stem cell lines.

These embryonic stem cells (ESC) were derived by culturing blastocyst on a feeder layer (mitotically inactivated fibroblasts were used) in a specific cell medium (Evans, 1981; Martin, 1981).

It is important to distinguish ESC of the inner cell mass from the epiblast-stem cells (EpiSC) , since it has been demonstrated that, although both are embryonic stem cells, only the first have the ability to colonize the germ line when injected in recipient blastocysts. Epiblast-stem cells are derived from the epiblast at the post-implantation stage and can contribute to chimeras but not to the germ line; they are less pluripotent compared to inner cell mass stage (Zhou et al., 2010). It has been demonstrated that appropriate synergy of epigenetic and signaling modulations could convert EpiSC to the earlier mESC pluripotency state, providing new insights into mechanism of pluripotency regulation (Zhou et al., 2010).

Mouse embryonic stem cells (mESC) are pluripotent stem cells with the capability of self-renewal and differentiation; *in vitro* they can be propagated as a homogeneous, uncommitted cell population for several passages without losing their pluripotency and their chromosomal stability (Prelle et al., 2002). Mouse ESC are cultured *in vitro* on a feeder layer of mouse embryonic fibroblast (MEF); MEFs must be inactivated prior to use by using γ-irradiation or mitomycin-C treatment; both methods produce feeder layers suitable for the maintenance of undifferentiated cells. Mouse ESCs *in vitro* show distinct morphological features: they grow in compact multilayered colonies, have a high nuclear:cytoplasm ratio and show alkaline phosphatase activity, a phenotypic marker of pluripotent stem cells, shared by induced pluripotent stem cells (iPSCs), and embryonic germ cells (EGCs). mESCs express high conserved membrane markers, such as SSEA-1 (stage-specific embryonic antigen-1) (Solter and Knowles, 1978), a surface protein that can selectively bind or adhere to other signal molecules. SSEA-1 was identified by using a monoclonal antibody, recognizing a defined carbohydrate epitope, and is involved in controlling cell surface interactions during development *in vivo*. This marker

decreases during differentiation in mouse, while increases in human ESC (Zhao et al., 2012). mESCs also show as markers of pluripotency some transcription factors, that are crucial for gene regulation; some of these are usually present in an inactive form, but are activated when are generating specific activation signals. The most important transcription factors involved in maintaining pluripotency in mouse are OCT4, Sox2 and Nanog. OCT4 (octamer-binding protein 4) is a member of the Oct family of POU transcription factors; it has been demonstrated that the POU domain is critical for the function of DNA transcription's regulation, while the region outside the POU domain is not of fundamental importance for DNA binding and has little sequence conservation. A different expression of OCT4 is been observed during embryogenesis, when the inner cell mass differentiates into epiblast and hypoblast in the first days of development; its expression is limited to pluripotent stem cells and germ line cells (Zhao et al., 2012). SOX-2 is a member of the SOX gene family that belongs to the HMG (high mobility group) box transcription factors group, and interacts with POU domain proteins. Unlike OCT4, SOX-2 is expressed in pluripotent stem cells as well as some unipotent and multipotent stem cells (Liu et al., 2013).

Nanog is a transcription factor that works in maintaining pluripotency as well as self-renewal in mouse, and also in human ESC, and has also been described to be required for the maintainance of pluripotency in mouse EpiSC (Mitsui et al., 2003). Nanog expression is known to fluctuate in ESC, and different levels of Nanog seem to correlate with ESC capability to respond to differentiation promoting signals. Its expression is early down-regulated during differentiation, and is able to maintain self-renewal independently of the LIF/Stat3 pathway (Do et al., 2013; Medvedev et al., 2012; Zhao et al., 2012). If Nanog and Oct4 are essential to maintain pluripotency, LIF activation pathway plays an accessory role. mESCs *in vitro* are grown by using a specific cytokine, leukemia inhibitory factor (LIF), that is able to maintain the stemness of the cells. It is a soluble glycoprotein of the interleukin-6 (IL-6) family of cytokines, which acts via membrane-bound receptor signaling complex protein, to regulate cell

functions by using cell signal transduction. The LIF receptor (LIFR) is formed by two subunits: gp130, which is common to all the cytokines from the IL-6 family, and LIFR (or gp190), specific for LIF. Despite the JAK-Stat3 pathway is essential and sufficient to maintain pluripotency of cells mediating LIF signal, how the signal is transmitted to the transcription factors that mediate the pluripotency it is not yet clear. In mESC it has been demonstrated that the presence of LIF is able to activate an intracellular pathway by using different transduction ways (Niwa et al., 2009; Pan and Thomson, 2007)(**Fig 2**).

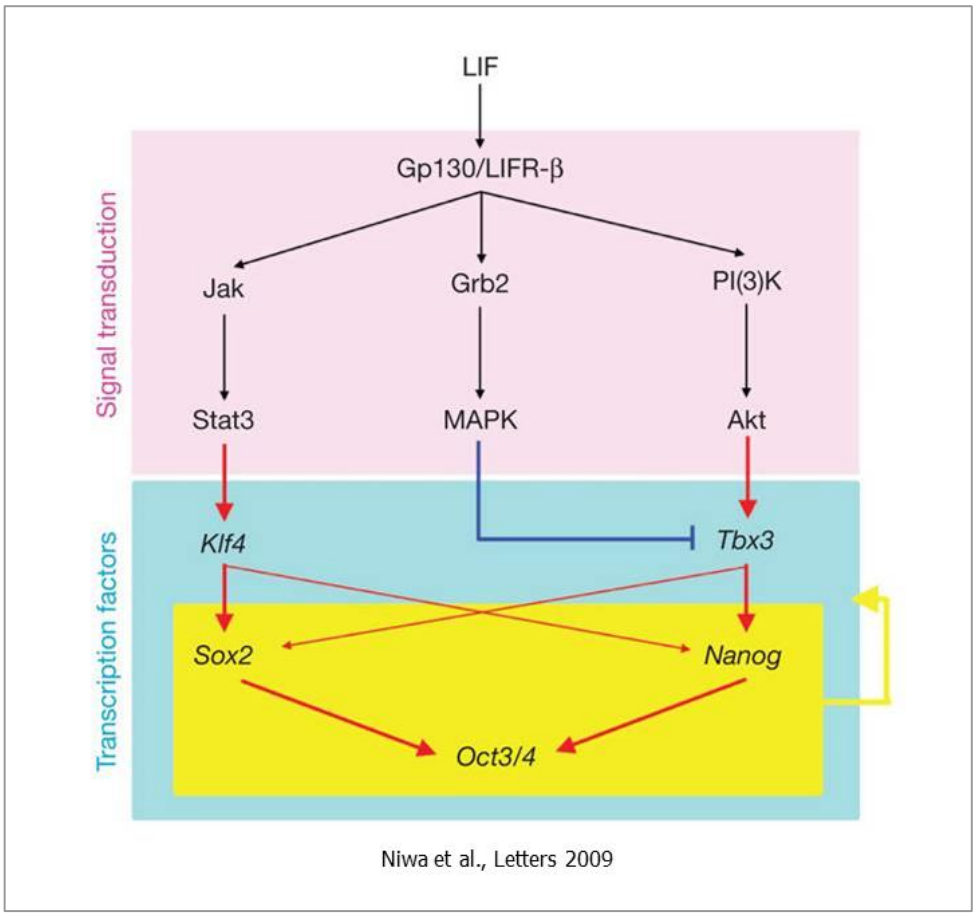

Fig. 2- Pathways of LIF signal in pluripotent stem cells. LIF signal is integrated into the core regulatory pathway using two parallel ways: the JAK-Stat3 way, that activates Klf4 and then Sox2, and the PI3K pathway that activates Tbx3 and then usually Nanog (Niwa et al., 2009). MAPK-kinase, on the other hand, prevents the up-regulation of Klf4 expression by controlling Tbx3 and Nanog.

LIF signal actives three pathways: JAK-Stat3, PI3K-Akt and MAPK pathway. Among them, the first one is activated exclusively by LIF signal; instead the other ones are also regulated by different intermediates. It has been show that LIF signal is integrated into the core regulatory pathway using two parallel ways: the JAK-Stat3 way, that activates Klf4 and then Sox2, and the PI3K pathway that activates Tbx3 and then usually Nanog (Niwa et al., 2009). MAPK-kinase, on the other hand, prevents the up-regulation of Klf4 expression by controlling Tbx3 and Nanog. In the absence of LIF, the activation of Stat3 or the overexpression of Nanog is sufficient to maintain the pluripotency and self-renewal. Stat 3 is activated by Janus Kinase 1 (JAK1), protein, and has been shown that STAT3 directly binds to the Oct4 and Nanog distal enhancers, to modulate their expression in maintaining pluripotency in mouse embryonic stem cell (Do et al., 2013). There is a relation between different transcription factors involving in maintaining pluripotency: in the cascade of events Tbx3 (deriving from PI3K cascade) and Klf4 (deriving from JAK cascade) are upstream whereas Nanog is downstream to Tbx3, to activate Oct4 expression. This complex regulation composed by parallel pathways and overlapping functions to control the self-renewal is necessary to maintain the pluripotency of cells under specific stimuli. It has been highlighted that the absence of IL-6 family members, or the inactivation of STAT3, can be sufficient to promote ESC to spontaneously differentiate *in vitro* (Wobus and Boheler, 2005).

Pluripotent stem cells usually are subjected to a series of assay *in vitro* e *in vivo* to clarify their stemness, as can be summarized in the **Tab 1**.

Pluripotency Assay	Purpose	Length of Assay	Nature of Assay	Strength/Definitiveness of Assay
Colony morphology	Verify ESC colony-like morphology of clustered, border-definied colonies.	10 minutes	*in vitro*	Low
Immunohistochemistry	Stain for standard pluripotency markers such as Oct4, Tra-1-60, Sox2, Tra-1-81, Nanog and SSEA.	1–4 days	*in vitro*	Medium
Real-time PCR	Detect and quantify expression levels of selected pluripotency genes; limited by gene number.	4–6 hours (from RNA extraction to RT-PCR	*in vitro*	Medium-High
Embryoid body formation and analysis	Test differentiation capability of PSCs to tissues of all 3 germ layers *in vitro* or *in vivo*; should be coupled with relative quantification expression of gene-expression from the three germ-layers, which can be done by RT-PCR. Genes include: Nanog, Oct4, Sox2, and Klf4 (pluripotency markers); Ncam and NeuroD (ectodermal markers); Runx2, HNF4a, and Nkx2.5 (mesodermal markers); and Sox17, Albumin, Glut2, and Insulin (endodermal markers).	2–3 weeks	*in vitro* *or* *in vivo*	Medium-High
Microarray	A comprehensive measurement of gene expression levels.	1–2 days	*in vitro*	Medium-High
Teratoma formation	Test differentiation capability into all 3 germ layers *in vivo*.	1–2 months	*in vivo*	High

Zhang et al., Stemcell book 2012

Tab 1- Most common assays used to show the stemness of stem cells.

In addition to the morphology of the colonies *in vitro* and markers of pluripotency, that are generally investigated through immunoistochemistry assays and molecular biology techniques, such as staining for standard pluripotency markers and real-time PCR, another common test used to show the pluripotency of mESC is the ability to produce a teratoma *in vivo*. Teratoma demonstrates the ability of cells to differentiate into the three germ layers (Przyborski, 2005). Teratomas are benign tumors characterized by rapid growth *in vivo*, and consist of multiple tissues organized often in quite complex structures. There are several protocols to describe the procedures that must be observed for the injection of stem cells in the mouse in order to obtain teratomas, and mice with a compromised immune system are used (NOD/SCID/γ-common chain NSG, or nude mice) to avoid rejection. mESCs can be injected subcutaneously or intraperitoneally, only in some cases the preferred site of injection is the kidney capsule or testes. Four to six weeks post-injection teratomas will become palpable (Zhang et al., 2012) and removed (**Fig 3**).

Zhang et al., Stemcell book 2012

Fig 3 - Teratoma formation in immune-compromised mouse. mESCs can be injected subcutaneously and four to six weeks post-injection teratomas will become palpable .

Analyzing the teratoma from a histological point of view, can be observed that cells derived from all three sheets of embryological derivation will be present.

The same *in vitro* demonstration of pluripotency of stem cells can be obtained by embryo-body (EB) formation (Marti et al., 2013). When mESCs are grown in absence of LIF *in vitro*, or without a feeder-layer that permits the maintenance of stemness, ESCs tend to differentiate spontaneously and to aggregate in a three-dimensional formation called embryo-body, a structure that facilitates multicellular interactions. EB consists of endodermal, ectodermal and mesodermal tissues and can be considered as a model of *in vitro* differentiation (Kurosawa, 2007). Different methods to generate EBs have been described, and the most common protocols are the suspension culture in bacterial-grade petri dishes, culture in methylcellulose semisolid media, and culture in handing-drops. The EBs obtained can then be analyzed to assess the presence of cells

pertaining to the three germ layers, thus demonstrating their ability to differentiate *in vitro.*

Finally, another assay to test the stemness of ESC *in vivo* is the injection in blastocysts to produce a chimera. Chimera refers to a single organism composed by two or more different population of genetically distinct cells. The contribution to the chimera organism by each parental cell type can vary in the various tissues and organs. The first experimental mouse chimera was done in the 1960s, by aggregating two or more whole 8-cells embryos, to obtain a single individual consisting of a mixture of parental embryos (Mintz, 1962; Tarkowski, 1961). After that, chimera organisms were produced also with tetraploid host embryos; tetraploid embryos were not able to produce viable offspring, but they have the ability to contribute to functional extra-embryonic tissues. When ESCs are introduced into mouse tetraploid embryos, they colonize the proper embryo and are able to give rise to the offspring, supported by extra-embryonic tissues deriving by tetraploid cells (Wang et al., 1997).

With regard to the cell cycle, ESCs show unusual features, composed by an S phase, a short G1 phase and the absence of the G1/S checkpoint. Cell division and proliferation are controlled by mechanism ensuring the accurate transmission of the genetic information from generation to generation. So far, despite the great progress in the knowledge of the cell cycle of ES cells, many regulatory mechanisms remain unknown (Abdelalim, 2013). Cell cycle in murine stem cell is rapid, estimated at 8-10 hours and is assumed to be similar to that of peri-implantation embryos; mESCs have a truncated gap phases, and an unusually high proportion of asynchronously dividing cells are in S-phase (more or less 65%) when compared with G1 (more or less 15%). It has been hypothesized that proteins directly regulating DNA replication might also stabilize pluripotency, and it's possible that the high order chromatin rearrangements that also occur during S-phase in order to allow DNA replication to proceed, could provide epigenetic modifications that stabilize the pluripotent state (Hindley and Philpott, 2013).

1.2. GENE THERAPY

The term "gene therapy" potentially includes different approaches to cure inherited diseases. The US Food and Drug Administration (FDA) defines gene therapy as "products that mediate their effects by transcription and-or translation of transferred genetic material and/or by integrating into the host genome and that are administered as nucleic acid, viruses or genetically engineering organism" (Wirth et al., 2013).

The possibility to use viral agents as "carriers" of genetic material into cells in order to develop gene therapy protocols has its roots in the early 60s. Since then, there have been many advances on the topic, and the early 90s saw the development of tools for recombinant DNA technologies, to develop innovative approaches of gene therapy using recombinant DNA (Cotrim and Baum, 2008; Mali, 2013).

Gene therapy can potentially be applied during embryonic (germ-line therapy) or fetal life or only into specific somatic cell (somatic gene therapy); using the second approach, DNA correction would not be passed into subsequent generation (Orkin, 1986). The difference is substantial, in that current legislation, for human individual, allows only somatic gene therapy.

The two main methods can be used for gene therapy are the *ex vivo* and *in vivo* approach. In the *ex vivo* approach cloned genes are transferred *in vitro* into cells explanted from the body, and in this way the rejection by the immune system can be prevented since the cells are autologous. Gene therapy *in vivo* is carried out in all those cases in which cells cannot be manipulated *in vitro* or removed and re-implanted, such as those of the brain or heart, and also more difficult to apply. In this case, the correction procedure is performed in the body, via a suitable vector, administered either locally or systemically.

Somatic gene therapy represents the main topic of several lines of research, and the goal is to obtain a therapeutic effect by introducing the genetic material in the cells to be corrected, without causing toxicity to these cells. With this aim, it is necessary to know the genetic damage to be replaced, the position of the rearrangement that involves the DNA sequence and the molecular characterization of DNA damage.

1.3. GENE THERAPY APPROACHES TO THE CORRECTION OF HEREDITARY RECESSIVE DISESASES

Gene therapy can be generally applied using three different approaches: gene addition, gene correction and gene knockdown.

The gene addition therapy is applicable to diseases caused by gene recessive mutations in a single gene: it involves providing cells with an exogenous copy of the wild-type allele, whose expression gives the normal and functional protein, and this mechanism should rescue the cells from the disease phenotype. The rationale of this approach is straightforward, and could be applied to most recessive diseases and to those due to haploinsufficiency. There are two ways to implement this approach: either by insert a copy of the gene transcript (essentially a cDNA) randomly in the genome or by targeting the abnormal *locus* reconstituting the correct sequence (homologous recombination).

A different strategy is needed for the diseases caused by dominant genetic mutations; in this way to give an extra-copy of the correct allele cannot be enough, so other gene therapy approaches, able to destroy or inactivate the

abnormal protein or its production, have been investigated by using a knockout approach.

1.3.1. FIRST GENERAL APPROACH: random inserting a gene by viral vectors

In theory, every procedure allowing the random insertion of a DNA fragment into the affected cells could work. However, today mainly methods based on modified virus vectors are used and will be briefly reviewed here.

First of all, the viral life is divided in two times, the infection time and the replication time. The first one is the introduction of the viral genome into the cells; the second one is given by the production of regulatory protein and the expression of viral genes. Using viruses for gene therapy is necessary to engineer a viral vector to carrier a therapeutic sequence in place of viral genome. The virus is rendered unable to reproduce themselves, to prevent the spread of recombinant viruses (defective virus); its genome is engineered with recombinant DNA techniques, and the individual plasmids are co-transfected into particular cell lines capable of producing recombinant viral particles (packaging lines). These lines are able to complement the defects introduced into the viral genome. This step is crucial to be able to control any hope of recovering from the virus in active viral particles (Kay, 2011).

The concentration of carriers is measured by the expression of the title. The main advantage consists in the high transduction efficiency (up to 100% of the cells). Once produced the recombinant viral DNA, this may be analyzed first, and then used. In gene therapy is important measure the amount of viral particles used in order to obtain the best efficiency of infection even with a low toxicity to the cells that are infected. Unfortunately, most of viruses used are integrated in the genome of the target cell in a random manner; this leads to incontrollable mutational events in the target genome. This evidences led over

the years to develop recombinant viral DNA with the lowest possible toxicity for the cells (Boeckle and Wagner, 2006; Huang and Kamihira, 2012).

The most used viruses for gene therapy in the last ten years can be grouped in three most important classes: retroviruses, which include lentiviruses and oncoviruses, adenoviruses and adeno-associated viruses.

Classical retroviruses are able to infect only dividing cells; they are lipid-enveloped particles composed by homodimer of linear single-strand RNA sequences (no more than 11 kb). They integrate themselves randomly in the target genome. (Gabriel et al., 2011b). Because of their RNA-sequence, once into the infected cells they have to be retro-transcribed into linear double-strand DNA to integrate permanently in the genome. The virus then exploits the normal processes of transcription and translation in order to express the viral genes. Different types of viruses are included in the retrovirus family: oncoviruses, lentiviruses and spumaviruses. In the recombinant DNA molecule inserted into the structure of the virus, some viral genes are replaced with the cDNA of the target gene; in this way the vector is not able to produce the proteins necessary for another cycle of infection. So the carrier is rendered defective for replication; viral proteins necessary for initial viral infection are given in trans by packaging cells or helper virus. Generally, up to eight kb of exogenous DNA can be inserted and expressed by the viral vector. One of the first promising results came in 2000 when the ability to cure SCID patients using retro-virally transduced autologous bone marrow was demonstrated; despite the initial enthusiasm, some of these patients developed leukemia after treatment, because of high activation capacity exerted by the LTR regions towards genes flanking the insertion site (Kay, 2011). Among retroviruses, lentiviruses are now the most used in gene therapy; their genome structure is more complex because is composed also by some regulatory genes, necessary for genome expression. Unlike the classical retroviruses, lentiviral vectors are able to target also non-dividing cells and have a LTR that lack a strong enhancer (Sakuma et al., 2012). The first vector was obtained by engineering

the HIV-1 virus to transduce lymphocytes (Pluta and Kacprzak, 2009). It has been show that lentiviral vectors efficiently transduce differentiated epithelial tissues of human, rodents and other species, but at the same time the safety of this approach has yet to be definitively established (Kay, 2011). Besides, both the need for retro-transcription step of the genome sequence to DNA and the limited size of the construct that can be inserted in place of the viral genome, make these carriers still subject to debate (Kay, 2011).

Adenoviruses are characterized by non-enveloped icosahedral structure, and are composed by linear double-stranded DNA genome of 30-40 kb. They are essentially composed by two different parts, the core and the capsid. The genome is about 36 kb, composed by coding regions for genes expressed early and late, flanked by inverted terminal repeat (ITR) that are used as replication origins. Adenovirus is able to infect both dividing cells and not dividing cells, and after the cell infection the virus does not integrate into the host genome but remains episomal. This feature gives greater security to be used in gene therapy (Noureddini and Curiel, 2005; Noureddini et al., 2006). Recombinant adenovirus is constructed by using DNA molecule of interest inserted in the viral genome loci; the first adenoviral vectors used had a deletion that rendered the virus defective for replication. Over the years, the high applicability shown by these carriers has led to engineer their genomic sequence in order to maintain the high infective capacity while getting more manageable vectors from a safety point of view, passing from the vectors so-called first-generation to those of the third-generation. Among the latter, a class of recombinant adenoviruses such as Helper Dependent or Gutless is currently used as therapy vector. In them, all viral genes have been deleted leaving only the ITR regions and the packaging sequence to the left of the gene of interest, while the replicative functions are provided in trans by a "donor", such as an helper virus. In this way it's possible to keep the structure by replacing the viral genes needed for replication with the genes of interest that have to be between 100 bp and 36 kb in length. The disadvantages are that they have transient expression and, and *in vivo* show

high immune response. In fact, they can induce toxicity at the concentration required to obtain a therapeutic effect (Kay, 2011).

Adeno-associated viruses (AAV) are DNA single-stranded viruses, non-pathologic, that need a helper virus for replication and for the completion of their life cycle. Their genome structure is very simple: they are composed by two genes, *cap* and *rep*, respectively deputies to viral replication and packaging of the genome, and two inverted terminal repeat regions (ITR). Adeno-associated viral vectors are able to infect both dividing and non-dividing cells, and are able to remain episomal. This feature makes them a good tool for applications in the field of gene therapy (Dismuke et al., 2013). Also in this way the viral genome in the hybrid vectors is replaced by genomic sequences of interest which can then be transported within the cell, the only limit being the size. In fact, the insert size is around 4 kb (Coura Rdos and Nardi, 2007). The main advantages are the fact that AAV *in vivo* are not pathogenic to humans, are easily obtained with high titers and allow a high efficiency of gene transfer; on the other hand, can convey only small molecules and can cause a high immune response. To overcome this last limitation, various strategies are being tested (Hareendran et al., 2013).

Another family of viral vectors widely used for clinical trials related to the central nervous system is the Herpes Simplex Virus (HSV-1). These have the capacity to establish latent infections in certain districts of the organism, and this capacity is exploited in a controlled manner in order to activate the expression of genes inserted into their genome.

1.3.2. THE SECOND APPROACH: targeting the abnormal locus by homologous recombination (HR) means

Historically, homologous recombination (HR) in mammals has been developed in mouse ESC. The beauty of this technique is that it is possible to perfectly correct the DNA abnormality, restoring the original wild type DNA sequence (Babinet and Cohen-Tannoudji, 2001; Capecchi, 1989; Mansour, 1990). In this way, the gene will be under the control of its own normal regulatory sequences, eliminating the bias of a random insertion and avoiding the silencing mediated by the cell recognition of viral sequences. In addition, any interference by exogenous viral sequences, which have been linked to cancer, will be prevented.

The HR approach is known also as gene targeting, and has been used for several years to manipulate mouse genome to create knock-in and knock-out mice (Wong and Chiu, 2011). This strategy for gene therapy is better than supplying an extra copy of the gene, because the correction at the endogenous genomic *locus* leads to a functional gene in its natural environment with a natural regulation of expression. Furthermore, when cells are corrected in the right target sequence, the corrected gene will be expressed indefinitely (Templeton, 1996). HR is based on pairing of DNA sequences, exploiting the homology of the complementary regions; this interaction therefore allows the substitution of the nucleotides of the genomic region damaged with a correct DNA sequence (**Fig 4**).

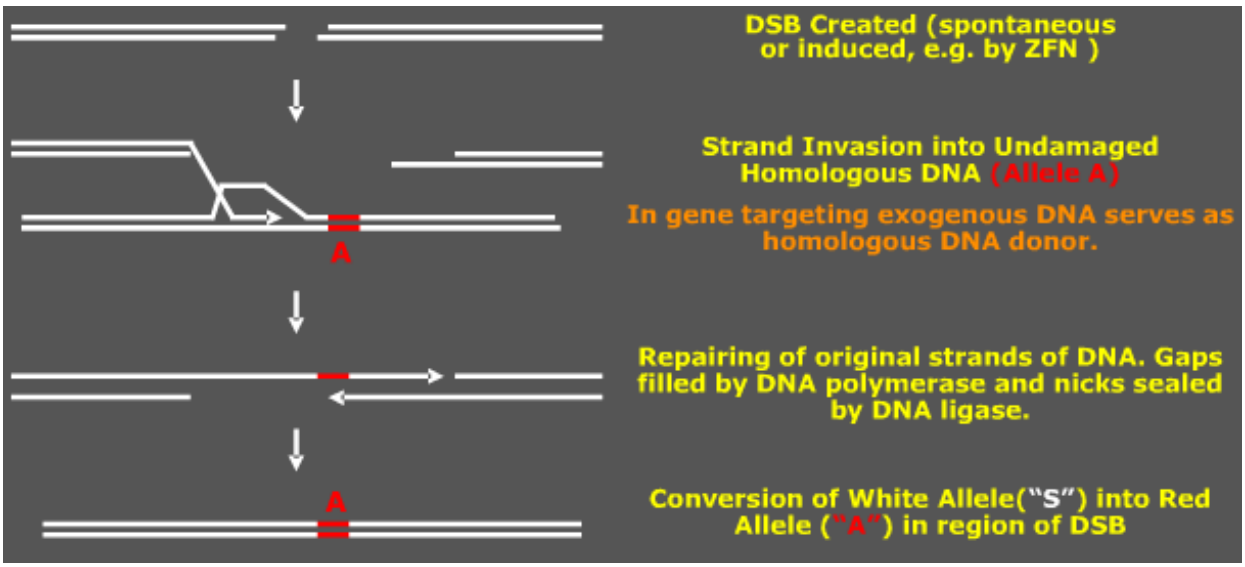

Fig 4 – Schematic homologous recombination mechanism. A double strand break is identified and replaced by using homologous DNA donor as template; in this way a damage can be corrected (red sequence).

Different vectors types are used for HR, and are all constructed to allow binding of the homologous regions to the target site of interest, and to replace the mutated endogenous region with an exogenous normal fragment. Vectors usually present also a marker of resistance, to select cells *in vitro* where the insertion of the correct sequence has occurred. It has been shown that in ESCs the frequency of HR is much higher than in somatic cells, and for this reason mESC became the preferential material for this kind of genetic manipulation. HR in mESCs led to the generation of thousands of knockout and knock-in mice, but, so far, has not been used in clinical trials, due to the extremely low efficiency of the HR in somatic cells.

1.3.2.1. New technologies for homologous recombination

Since the homologous recombination occurs even *in vitro* with very low efficiency, in the last few years mechanisms of gene targeting to increase the recombination efficiency were developed. The rationale of these advances was the idea that, by causing a breakage in the DNA double helix, within the region of interest increases HR events.

The most common approaches to gene targeting are Zinc-Finger Nuclease (ZFN), Transcription Activator-Like Effector nuclease (TALEn) and Clustered Regularly Interspaced Short Palindromic repeats (CRISPR); all of them are able to identify a specific DNA region in the genome and thanks to a linked nuclease these complexes are able to cut in the preselected region. It has been shown that this approach increases the efficiency of homologous recombination up to 10^{-1} 10^{-6} (Mali and Cheng, 2012).

1.3.2.1.1. Zinc Finger Nuclease (ZFN)

The zinc-finger structure was described for the first time more than 20 years ago as repeated zinc-binding motifs, containing cysteine and histidine ligands; in the structure of the protein one or more zinc residues are necessary for the stabilization of conformation. Since that, different zinc-binding proteins that result from variations of the classic motif Cys2-Hys2 have been identified. They have the most variable functionality, from DNA or RNA binding to protein-protein interaction, and are all characterized by high binding specificity to a target region. This structure has been engineered to obtain zinc finger nucleases, hybrid proteins composed by a DNA-binding domain and a non-specific cleavage domain (usually is used Fok1): the first one is able to bind specific DNA sequences exploiting the specificity of interaction DNA-protein, while the second one is able to produce double-strand breaks in that genomic region (Carroll, 2011; Chou et al., 2012). Each motif, consisting of

approximately 30 amino acid, is able to recognize 3 nucleotides and is composed by an α-helix and two β-antiparallel sheets; the contact is made by the side chain of the α-helix (**Fig 5**).

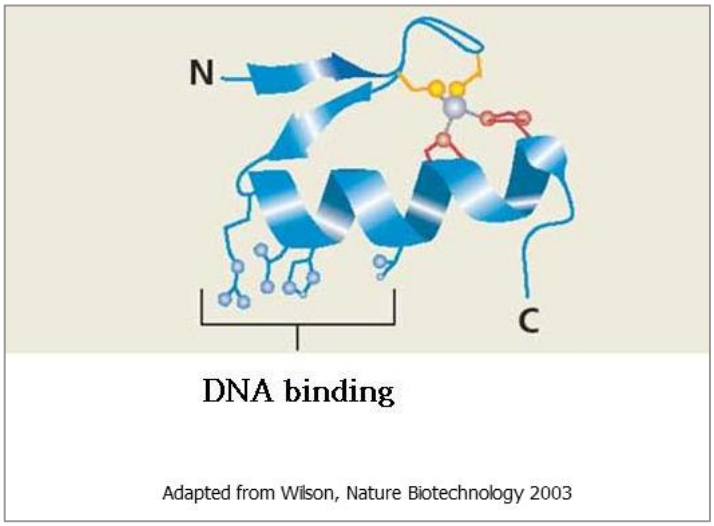

Fig 5- Structure of the ZFN motif. The protein presents two anti-parallel β-sheet and one α-helix; the side chain of the α-helix is responsible of the DNA-protein binding.

The associated FokI nuclease works as a dimer: two adjacent and independent binding events (reverse and forward) must occur both in the correct orientation and with appropriate spacing, allowing dimer formation (**Fig 6**). To do that, two independent ZFNs have to bind DNA in the correct orientation and position. The dimerization of the nuclease triggers its activation and thus the production of a double-strand break in the genomic region comprised between the two target sites of ZFNs (**Fig 6**).

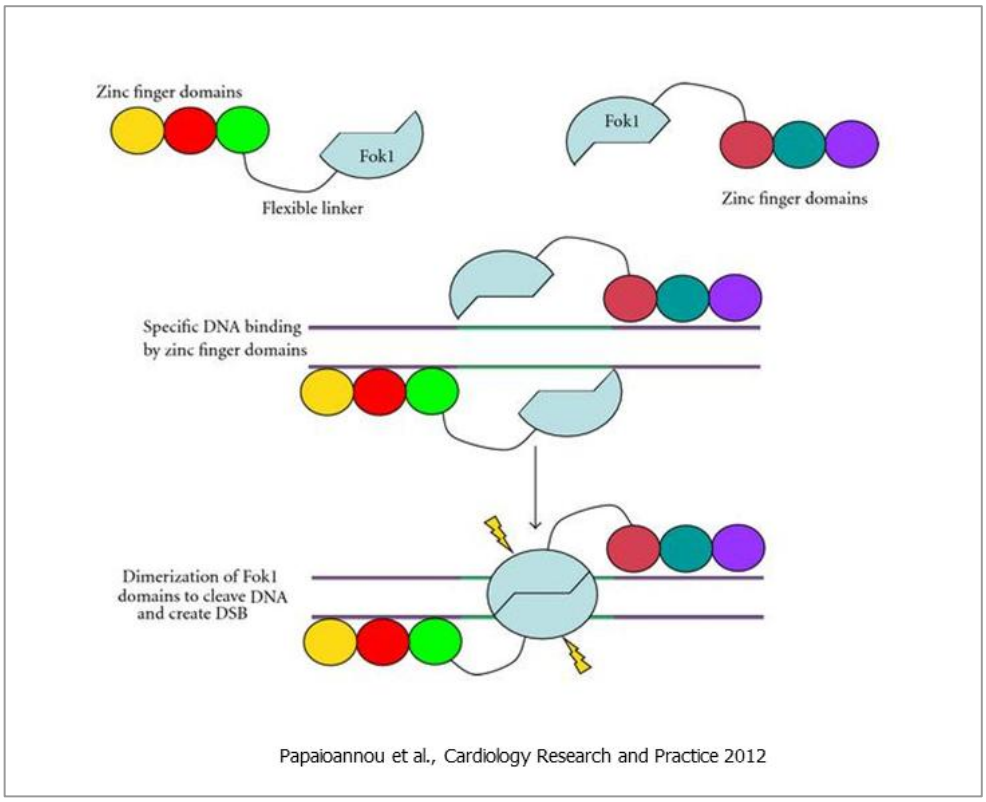

Fig 6 - Mechanism of action of ZFN motif. Each ZNF, shown as a colored circle, recognizes 3-4 nucleotides. In this way, a ZNF motif is able to link a specific DNA sequence. FokI nuclease is activated by dimerization, so the customized ZNFs work in pair. The FOKI dimerization and its activation permits the production of a double-strand break in the region of interaction.

Early studies on ZFN used three motif-array to bind a 9-bp target, given a cut specificity given by target region of 18bp. It has shown that a target site of 9-18 bp is sufficient to target unique sequence in complex genome. More recently, studies using ZFN have been conducted using up to six ZFN-motifs, to increase the specific of break (Urnov et al., 2010). Furthermore, using this approach bi-allelic modification at the same time can be created. For many years ZFNs have been the main approach for gene targeting; despite the high affinity that characterizes the method, it has been shown that is sufficient for the DNA binding and cleavage only 60% homology of putative target region. This limits the use of the technique, because of the side-effects of off-target breaks. Two recent studies have showed that ZFNs cleaved off-target sites in

genome besides the *in vitro* predicted DNA sequence, and this might cause chromosomal instability and genotoxicity (Gabriel et al., 2011a; Pattanayak et al., 2011). In addition, repair of ZFN cleavage sites might lead to frequent mutations (Cathomen and Joung, 2008; Lombardo et al., 2007) and abnormal integrations of donor DNA (Hockemeyer et al., 2009). Nevertheless, using this approach the ability to correct DNA mutations associated with SCID (severe immunodeficiency), hemophilia B, sickle cell disease and anti-trypsin deficiency has been demonstrated. Furthermore, studies about inactivation of genes involved in the onset of Parkinson desease and HIV / AIDS have been reported (Gaj et al., 2013b).

1.3.2.1.2. Transcription Activator-Like Effector Nuclease (TALEn)

The transcription activator-like effector (TALE) family in plants forms a subset of proteins involved in the modulation of host gene expression; in fact pathogenic bacteria have developed this strategy to colonized host plants.
The central region of these proteins shows the most fascinating and versatile feature of TALE. It is composed by a series of nearly identical 34/35 amino acids modules repeated in tandem (**Fig 7**); the hyper-variability is given by residues in positions 12 and 13; it is also referred to as the Repeat Variable Di-residues (RVDs), and every motif of the repeat domain recognizes only one nucleotide and this provides a greater binding specificity than zinc-finger protein.

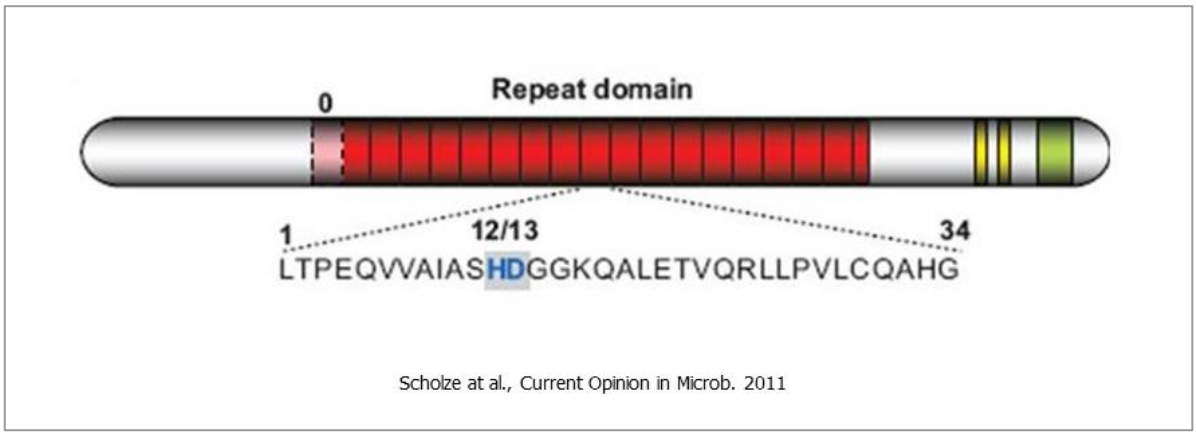

Fig 7 - Structure of the TALE protein. The repeat domain involves the central region of the protein and is composed by amino acids motifs repeated in tandem.

TALEn are artificial complexes composed by the fusion of a transcription activator-like effector (TALE) DNA binding domain and an endonuclease, FokI (N) at the C-terminal end. In this way it is possible to take advantage of the high specificity of DNA-protein binding to create double-strand breaks by using the nuclease activity, in a way similar to ZFN technology (**Fig 8**). The mechanism of genome editing is the same: two anti-parallel TALEN (forward and reverse) bind DNA and permit the dimerization of FokI nuclease; the only target limitation seems to be the requirement that the binding site have to start with T (Gaj et al., 2013a). It has been highlighted that a spacer of 12–15 bp between the two target half-sites is optimal for high TALEN cleavage activity (Mussolino et al., 2011).

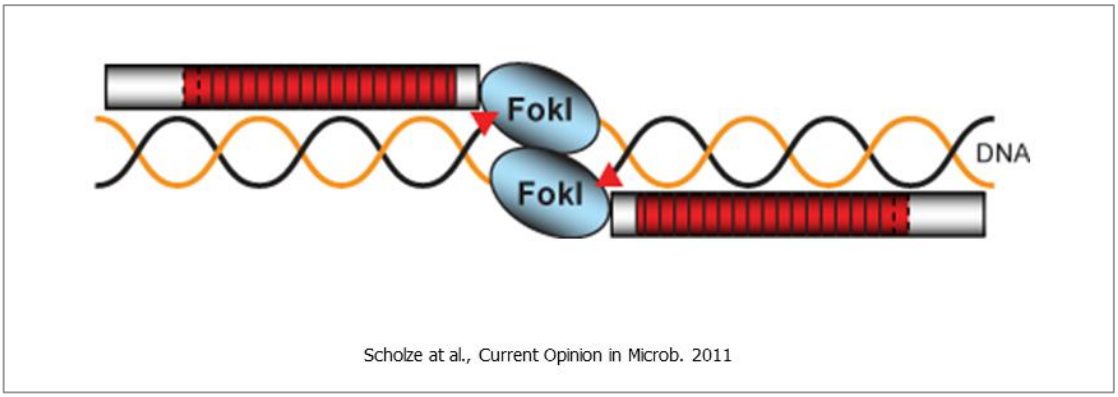

Fig 8 - TALEn genome editing mechanism. Left TALEn and right TALEn, each one linked to a FOKI nuclease, bind specific sequence DNA and cause dimerization of FOKI nuclease; its activation causes a double strand break in the region of interaction.

Repair of double-strand DNA breaks induced by TALEn can be exploited to induce targeted insertion/deletion mutations (by non-homologous end-joining-mediated repair) or specific substitutions or insertions (by homology-directed repair). Several groups have reported advances in genome editig using TALE-based engineering in different organisms, such as zebra fish, rats, yeast, mouse and man. For instance, TALENs have been used to achieved gene targeting at sites that are usually refractory to HR in mouse ES, such as Y-chromosome genes (Wang et al., 2013). Recent works *in vivo* has shown that injection of TALEn mRNA into fertilized mouse oocytes can give a high percentage of pups with targeted deletion with efficiency between 10-77%. *In vitro*, using this technology, it has also been possible to construct cell-based disease models, using somatic cells or induced stem cells (IPS). Also for this technology, there is a possible toxicity given by the off-target break in the genome; a recent paper (Mussolino et al., 2011) has shown that for CCR5 human *locus*, TALEN revealed only minimal off-target activity as compared to the corresponding ZFN, suggesting that the TALEn platform enables the design of nucleases with single-nucleotide specificity.

1.3.2.1.3. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

More recently a new molecular RNA-interference mechanism has been discovered in bacteria, and it has been exploited to be used as a gene targeting mechanism in mammals, also in this way mimicking what occurs in nature. In fact, many bacteria and Achaea protect themselves from viruses or other genome invasion by using a genetic interference pathway. To do that, they use short RNA molecules to direct degradation of foreign nucleic acids; this protection mechanism is called "Clustered Regularly Interspaced Short Palindromic repeats" (CRISPR) associated protein (Cas) system (Richter et al., 2012; Szczepankowska, 2012). Is not hard to see how this complex mechanism can be exploited for gene targeting. In fact, when Cas9, a protein of the type II CRISPR-Cas system, is complexed with two RNAs, trans-activating RNA (tracrRNA) and CRISPR RNA (crRNA), the sequence –specific endonuclease is activated causing a break in the target region genome. CRISPR *locus* is composed by short repeats, separated by non-repetitive sequence called spacers. In nature, they bound proto-spacer sequences providing a memory of past invasion. In this way, during invasion of bacteria, phage-derived spacers are inserted at one end of the *locus*, for the next immunization in the case of a subsequent attack. The CRISPR *locus* is flanked by CRISPR-associated genes (Cas) that encode protein components of the machinery (Cong et al., 2013). When CRISPR *locus* is activated, precursor crRNA is transcribed and then processed to produce crRNA mature, able to bound nucleic acid (**Fig 9**).

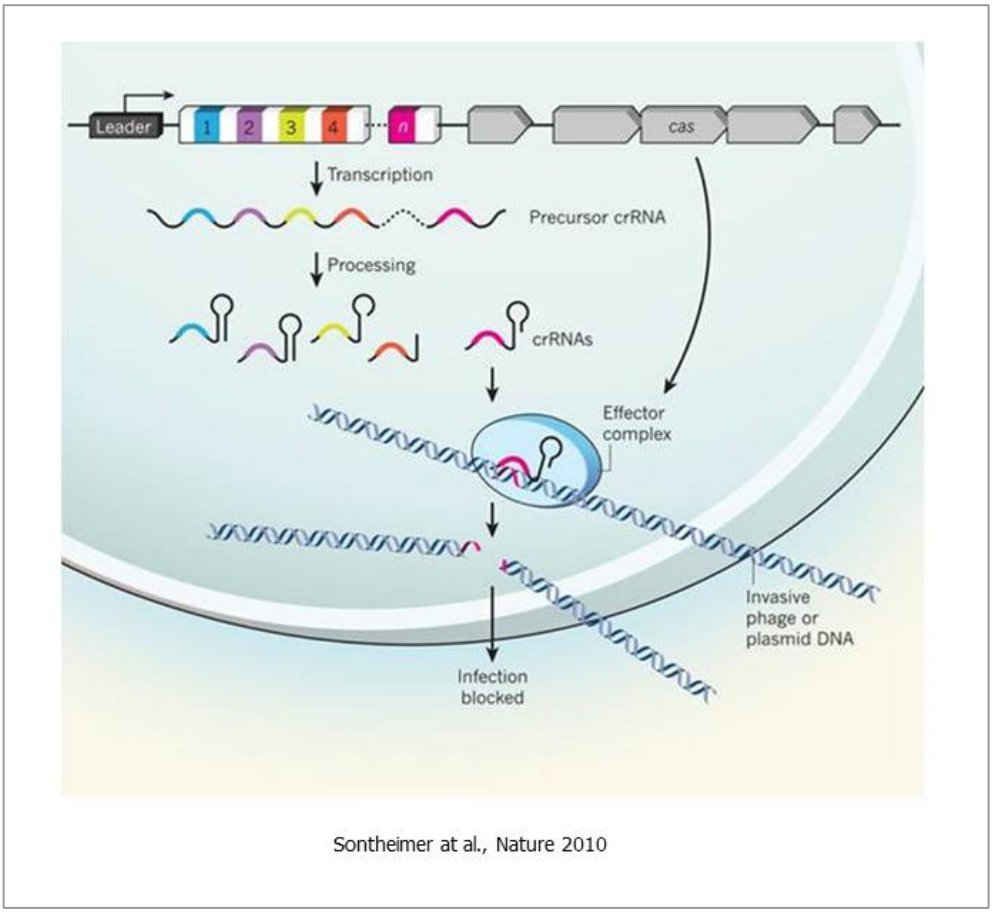

Fig 9 – CRISPR machinery. After foreign DNA infection, CRISPR array is activated and precursor crRNA transcripted. After its maturation, crRNAs bound Cas-proteins to became active and through the recognition of specific nucleotide sequences, bind and degrade DNA the invasor.

For complex functioning is necessary crRNA, Cas9 endonuclease and tracRNA; it has been show that tracRNA, transcribed by the CRISPR array, is necessary for the stabilization of the structure and for its maturation. The target recognition by Cas9 protein requires a seed sequence within the crRNA and a protospacer-adjacent motif (PAM) sequence upstream of the crRNA binding region. For the identification of target region by the mature crRNA it has been shown that is necessary and sufficient a 20bp sequence (**Fig 10**).

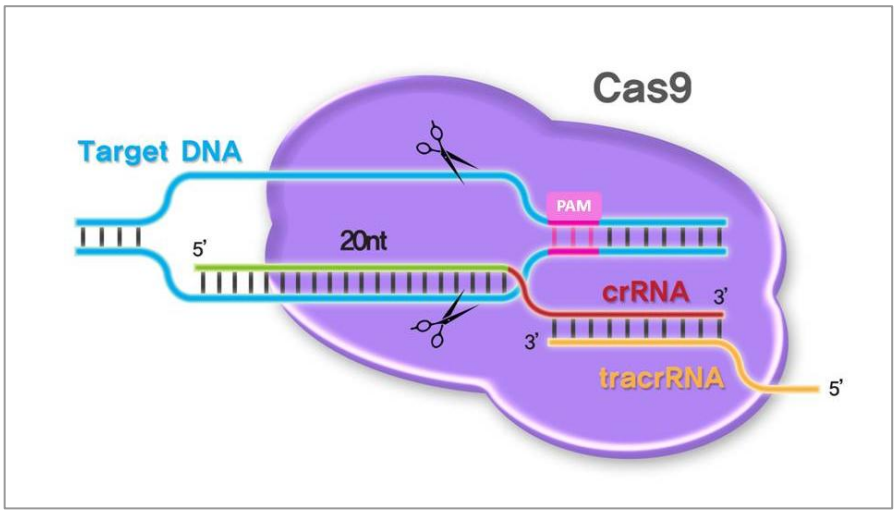

Fig 10 – Mechanism of action of CRISPR-Cas hybrid protein. A 20bp sequence in the target region is necessary and sufficient for the specific identification and binding of crRNA (green sequence). crRNA links a Cas9 nuclease (shown in violet), and after its activation it is able to cause doble-strand break in the region of interaction. tracrRNA links crRNA and is necessary for its stabilization. A PAM sequence (pink sequence) is necessary for the specificity of interaction.

In vitro, the interference machinery can be mimed by co-delivering an optimized plasmid expressing Cas9 endonuclease and crRNA component; crRNA and tracr-RNA transcripts are fused together to form hybrid guideRNA (gRNA), the active and stabilized form of RNA molecule able to bind genome sequence. gRNA binds Cas9 protein, which is activated, and so the active complex, driven by the 20 bp DNA sequence complementary in the target region and the flanking PAM sequences, recognized by the Cas9 itself, causes double strand break in the target sequence (Cho et al., 2013b). It has been shown that the CRISPR-Cas system produces double strand break three nucleotides upstream of the PAM motif in the target region (Ding et al., 2013; Ding, 2013; Feng et al., 2013). According to this model, CRISPR-Cas system is able to target and cleave any DNA sequence, only by redesigning the gRNA (Gaj et al., 2013a). Besides, Cas9 proteins cleave target region only in presence of gRNA, and do not cleave a region that lacks the target sequence (Cho et al., 2013a). *In vitro* the CRISPR-Cas mediated genome editing has been applied to human (in

particular stem cells), zebrafish and bacterial cells. In addition, experiments conducted in parallel on human pluripotent stem cells by comparing CRISPR-Cas9 system and TALEn system have shown that the efficiency of gene targeting for the first is higher than the second one (51-79% in CRISPR-Cas9 system versus 0-34%). In mESC, transfection of a plasmid expressing both the optimized Cas9 protein and a gRNA targeting three *Tet* genes, has given excellent results: all clones tested carried mutations in genes with an efficiency of 65% up to 81%, and more than 20% of clones carried mutations in all six alleles. To test the potential of multiple gene targeting cells have been co-trasfected with gRNAs simultaneously targeting five genes (including genes on Y chromosome); 10% of clones analyzed showed mutations in all five alleles (Wang et al., 2013). Excellent results have been obtained also *in vivo*: direct injection into fertilized egg of Cas9-mRNA and gRNA, targeting five genes, has shown an efficient production of mice bi-allelic mutated (around 80% of pups analyzed). Specific point mutations can also be obtained by giving a single-stranded oligonucleotide donor with homology to the target region together with Cas9 and gRNA. In this way specific point mutations were simultaneously introduced in two different genes in 6/10 founders analyzed (Wang et al., 2013). At the same time, the potential limit of the technique is the requirement for a N_{20}-PAM target sequence that could limit site selection; and also the off-target effects remain to be defined (Ding et al., 2013).

1.3.3. THE THIRD APPROACH: ARTIFICIAL CHROMOSOMES

To overcome the problems highlighted by the use of viral or non-viral systems, as a means for gene therapy, the 90s have shown the development of methods characterized by low toxicity and high expression of hybrid genomic sequences within a target cell, such as the human artificial chromosomes (HACs). HACs, human artificial mini-chromosome, can be developed by using two different methods: the top-down (engineered chromosome) and the bottom up approach (*de novo* artificial chromosome). Both methods give rise to a functional chromosome that replicates and segregates independently from the genome of the cell in which is inserted, as a "normal" chromosome (Kazuki and Oshimura, 2011; Larin and Mejia, 2002).

The top-down approach is based on two different strategies: the telomere-associated chromosome fragmentation and the radiation induced chromosome breakage. In the first approach, chromosome manipulation is generally done in intermediate host cells, and subsequently the chromosome of interest is transferred into a hybrid cell line by whole cell fusion; it's important to have a selectable marker gene as close as possible to the centromere of mini-chromosome for its subsequent selection. Different groups have used this methodology to insert into cells genomic sequences of interest. It was observed that within the HAC structure a well-defined insertion site for the exogenous gene of interest is necessary. One of the fundamental steps in the development of the methodology was reached in 2004 by Oshimura's group, who built HAC 21 Δ–pq telomere-directed breakage of human chromosome 21 in DT40 cells. Innovation was brought by the insertion of a loxP *locus* with a "neo" gene without the promoter in HAC structure, in this way any vector carrying a loxP site and a promoter can restore neomicine-expression by using the Cre-loxP system. This points out that by using this method it was possible to exploit the well-defined genomic structure of the carrier and a targeted insertion of the

transgene of interest using the loxP system, as well as to maintain the structure of mini-chromosome stable over time (Oshimura and Katoh, 2008). Using the HAC21 Δ–pq vector, various studies, based on the insertion of specific genes within the cell, such as the erythropoietin gene in human fibroblasts, or the mediation cell lineage-specific transgene expression, have been performed (Kakeda et al., 2005; Otsuki et al., 2005; Ren et al., 2005), and all applications have shown great versatility and functionality (Oshimura and Katoh, 2008).

The bottom up approach is based on assembling an *de novo* chromosome by providing into the cells genomic sequences for its basic structure. Repeats of telomeric DNA sequence of a few hundred base pairs can generate *de novo* telomere at the end of the genome sequence. The bottom up approach was first demonstrated for the construction of yeast artificial chromosome (YAC) in *Saccharomyces cerevisiae*, and after that the technique was developed also in mammalian cells. For the first time in 1997 Willard group, by combination of synthetic human α-satellite sequence, human telomeric DNA sequence and randomly shared human genomic DNA inserted in human fibrosarcoma HT180 cells, achieved a stable exogenous mini-chromosome. Using this approach, the neo-formed chromosome ranges from 1 to 10 Mb in size and in the most cases is circular. It has been demonstrated the effectiveness of this vectors to vehicle sequences within the cell, such as the functional complementation of hypoxantine-guanine-phosphoribosyltransferase (HPRT) in human HPRT-deficient cells (Grimes et al., 2001) or the formation of HAC *de novo* carrying the guanosine triphosphate cyclohydrolase I (Ikeno et al., 2002). In the bottom-up approach most criticisms concern the lack of control over the genomic sequence which is formed; in particular, the lack of a direct relationship between the DNA inserted into the cell and the HAC sequence both in terms of size and composition. It could also happens that the inserted DNA does not participate in the formation of artificial chromosome, but instead is inserted randomly in the cellular genome, and also often HACs contain a non-predictable copy-number of gene of interest.

The use of chromosomal vector system shows different advantages, because they exist independently from the host genome and their inclusion does not involve insertional mutagenesis, can be transmitted to the progeny (in animal models) and can carry large genomic sequences including the regulatory regions. In fact, most eukaryotic genes are very large and their regulated expression often depends on genomic control elements; besides expression of most eukaryotic genes depend on the chromosomal environment in which they are positioned, and so engineered chromosomes might be used as an epigenetically regulated expression system.

In the bottom up approach one of the most delicate step is the regeneration of a stable and functional centromeric sequence, because until a few years ago, the exact structure of the centromeric region was unknown; the first results obtained on yeasts were used to construct the mammalian artificial chromosome. Studies in rodent cells have highlighted how the efficiency of transfer rates in recipient cells is around to $10^{-5}/10^{-4}$ (Kouprina et al., 2013). In both procedures to obtain an artificial chromosome, many advances have been made regarding their possible applicability as a carrier for gene therapy approaches, due to their safety and their ability to convey large molecules. For instance, gene therapy approaches for the replacement of dystrophin gene, the lack of which causes Duchenne muscular dystrophy, were conducted by conventional techniques, but have not led to good results because of the large size of the gene sequence; the use of HAC as a vector for the transport into diseased cells would be a safe and innovative approach that would exceed the size limit (Ren et al., 2006). Another important application of chromosome engineering technology is to generate animal model with aneuploidy syndrome to study the genetic basis of the disease and possible gene therapy approaches for their care (Oshimura and Katoh, 2008).

The major limitation of HAC technique, however, remains the transfer method into the recipient cells of interest, because the conventional transfection approach is inappropriated.

The most common transfer method to introduce HAC vector into recipient cells is microcell-mediated chromosome transfer (MMCT).

1.3.3.1. Microcell-mediated chromosome transfer (MMCT)

Microcell-mediated chromosome transfer (MMCT) is an approach to transfer whole or truncated chromosomes from a donor cell to a recipient one through microcell generation, their fusion with recipient cells and subsequent hybrid cell selection. MMCT allows the stable introduction of exogenous chromosomal material from a donor cell into a recipient cell, according to a cell fusion protocol. The technique was applied for the first time in the 70s, and since then many steps forward have been conducted to improve its protocol efficiency (Doherty and Fisher, 2003; Meaburn et al., 2005). The concept of chromosomal transfer is closely related to the concept of hybrid cell. Inter-specific somatic cell hybrids are obtained by the insertion in a somatic cell of one or more chromosomes belonging to another species, and have been used in several fields, one important example being the high resolution gene localization via radiation reduced cell hybrids.

The first cell fusion experiments dates back to the 50s, when fusing agents such as Sendai virus, were used to mediate the fusion of cell membranes. In the same years the possibility of obtaining multinucleated cells using mitosis-inhibiting substances, such as colchicine, was demonstrated. Multinucleated cells can be enucleated by using a cytochalasin B treatment and a subsequent centrifugation, in which the condensed chromosomes are "expelled" from the cell together with the nuclear and cell membrane, thus forming the micro-cells. Combining the protocols of multi-nucleation and whole cell fusion it has been possible to obtain hybrids each containing one or more chromosomes in addition to the normal karyotype structure of the recipient cell. At the end of the 70s the fusion of mouse microcells with human HeLa cells or Chinese

Hamster Ovary cells was reported, obtaining mouse-human and mouse-hamster hybrid chromosomal clones, respectively (Doherty and Fisher, 2003).

The MMCT rationale procedure is simple: when a cell line is exposed to colchicine for a prolonged period *in vitro*, the number of metaphase cells increases and the nuclear membrane reorganizes itself around one or more chromosomes. Micronuclei so formed can be brought out the cells after centrifugation in the presence of Percoll (to obtain a gradient) and a microfilament disrupting agent such as cytochalasinB, and in this way they can be collected. The gradient separation is based on differences of densities of nucleus versus cytoplasm under centrifugal force. Once obtained, the micronuclei are filtered through membranes with pores of different sizes, to select microcell containing only one chromosome; generally, the crude microcell preparation is filtered using 8, 5 and 3 υm nucleopore filters in sequence. Using this approach, only the smallest microcells, with complete elimination of whole cells and the 99% of karyoplasts, are obtained. These can be individually fused with a recipient cell which acquires the chromosome of interest, by using a fusogen agent, such as polyetilene glycol (PEG), that dissolves cell membranes, and phytohemagglutinin-P, that cause cell agglutination (Paulis, 2011) (**Fig 11**).

First of all, an heterokaryon is formed, a cell that contains nuclei from two different origins; after that, the fusion of the two nuclear membranes occurs and a single nucleus containing all the genetic material of the hybrid somatic cell, the synkaryon structure, is formed.

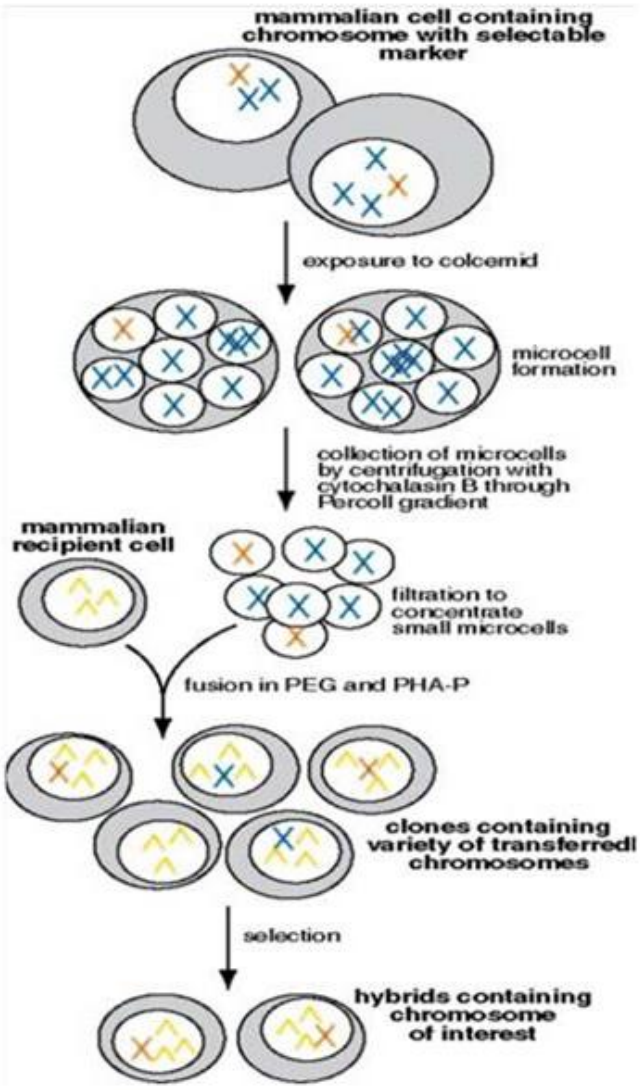

Fig 11 – Schematic representation of the MMCT protocol. Donor cell are exposed to colchicine *in vitro* for a prolonged period, then after Cytochalasin B treatment and centrifugation in a Percoll gradient, micronuclei are collected. They are fused with the recipient cell by using PEG and PHA-P to obtain hybrid cells. Clones containing different transferred chromosomes can be selected, to obtain only cells containing the chromosome of interest.

Two different methods for cell fusion, in suspension or in adhesion, can be used. The two protocols are similar but essentially revised according to the type of cells that are used. In the first protocol, the microcells preparation obtained is put in contact with the adherent cells, seeded at a specific density, and cell fusion is permitted through the use of fusogen agent that is added; in the second one this step is instead done in suspension after removal from the plate of both microcells and recipient cells (Killary and Lott, 1996).

By using the MMCT protocol, different clones carrying the normal diploid chromosome number plus the chromosomes provided by the fused microcell can be obtained. The somatic hybrids that are formed in a wrong way or the microcells that remain adherent to cells without fusion are not viable *in vitro* applying a selection system, as explained here below.

In fact, in order to follow the chromosome of interest during all the fusion steps, a marker gene, such as a resistance gene, which allows *in vitro* selection of the cell hybrids obtained, is needed. In this way hybrid cells grown under selective condition will survive only if they contain the preselected chromosome, in which the marker has been previously targeted to. For instance, the X chromosome, human 17 chromosome and mouse 11 chromosome can be easily selected by using the nucleotide salvage pathway complementation (HAT system). For other chromosomes, it is possible to insert a gene carrying the resistance for *in vitro* selection, such as the neo resistance. Generally, hybrid clones obtained retain the inserted chromosome as long as the selection medium is maintained *in vitro*.

The main limitation is that not all cell lines are able to micro-nucleate, and among these there are many rodent cell lines, such as Mc-Lox and A9 lines, and human foreskin fibroblast in early passages. It was observed that the micro-nucleation of many human lines is difficult, and in some cases the cells have low micro-nucleation efficiency and it is necessary to use a large amount of donor cells (Killary and Lott, 1996).

A normal donor cell line has to be fused with a cell line able to micronucleate, through a whole cell fusion, and a clone containing the desired chromosome is screened; this clone will be used for micronucleation. The whole cell fusion protocol is similar to that described previously. When two different cell-species are fused together, the resulting hybrid *in vitro* tends to lose preferentially most chromosomes of one of the two parental lines; for instance, in the human/rodent hybrid cells, most human chromosomes are generally eliminated. But it occurs that in some cases a single human chromosome is retained; in this way an inter-specific mono-chromosomal hybrid can be obtained (Paulis, 2011).

Despite the rationale of the method is very straightforward, micro-cell mediated fusion is a technique rather complex to be carried out, due to the very low efficiency rate ($10^{-4}/10^{-6}$) (Kakeda et al., 2005; Katoh et al., 2004; Ren et al., 2006). Different parameters may vary the efficiency of the protocol, including the concentration and the exposure time of colchicine, and the cell type of the donor line. Recently several methods have been studied in order to increase the efficiency of fusion *in vitro*, including the use of a lipid envelope of an inactivated hemagglutinating virus of Japan (HVJ), which has a function similar to PEG. It seems that this can increase the efficiency up to 3-8 times more (Kouprina et al., 2013).

1.4. LESCH-NYHAN SYNDROME

Lesch Nyhan (LN) syndrome was described for the first time by M. Lesch and W. Nyhan in 1964, and is characterized by a deficiency of the activity of hypoxanthine-guanine phosphoribosyltransferase (HPRT) enzyme (Torres and Puig, 2007; Torres et al., 2012). The clinical signs of the disease occur a little time after birth, and one of the first clinical evidence in infants are orange crystal in the diapers or crystalluria with obstruction of the urinary tract. These symptoms are associated with renal failure and acidosis associated with vomiting, until the appearance of the first psychomotor disturbances at 3-6 months of age, with delay in the sitting position and supporting the head, hypotonia and atetosic movements (Jinnah et al., 2006). Later, the manifestations of severe neurological disorders, mental retardation, a tendency to self-harm and hematological damage appear. In all patients with LN syndrome, the impairment of the pathway of purine metabolism causes an over-production of uric acid, which is associated to lithiasis and gout. Neurological damage can be manifested in rather different ways depending on the degree of enzyme deficiency present; there are, in fact, more mild forms of the disease that are defined Lesch-Nyhan variants, in which patients have only a partial deficiency of the enzyme (Torres et al., 2012).

1.4.1. HPRT GENE AND MUTATIONS

The disease is inherited as a recessive X-linked trait, in fact males are affected and women are generally asymptomatic carriers; the prevalence is 1/235.000-380.000 live births. LN disease is due to mutations in the HPRT gene, localized on Xq.26 in humans. The homology of the human gene compared to the ortholog mouse gene is very high, around 95% for the coding regions, encoding proteins that differ in only 7 amino acids positions (Boyd et al., 2000). Genes localized on X chromosome, in fact, in species of eutherian mammals are

highly conserved, so a mutated gene that leads to genetic disease in humans can be studied in another mammalian species, including the mouse. Exploiting the high degree of homology of the two genomes, and the known genomic localization of some genes present on the entire length of X chromosome, a comparative phenotype map of human and mouse chromosome has been built, to highlight the high conservation of syntenic blocks during evolution. Already more than twenty years ago five conserved blocks have been identified. After that, a more detail analysis of the structure was done. (Blaschke and Rappold, 1997). As now, twelve conserved segments in the chromosomal comparative map can be identified (**Fig 12**) (Boyd et al., 2000). Within a highly conserved region of the two genomes, is localized the HPRT *locus*.

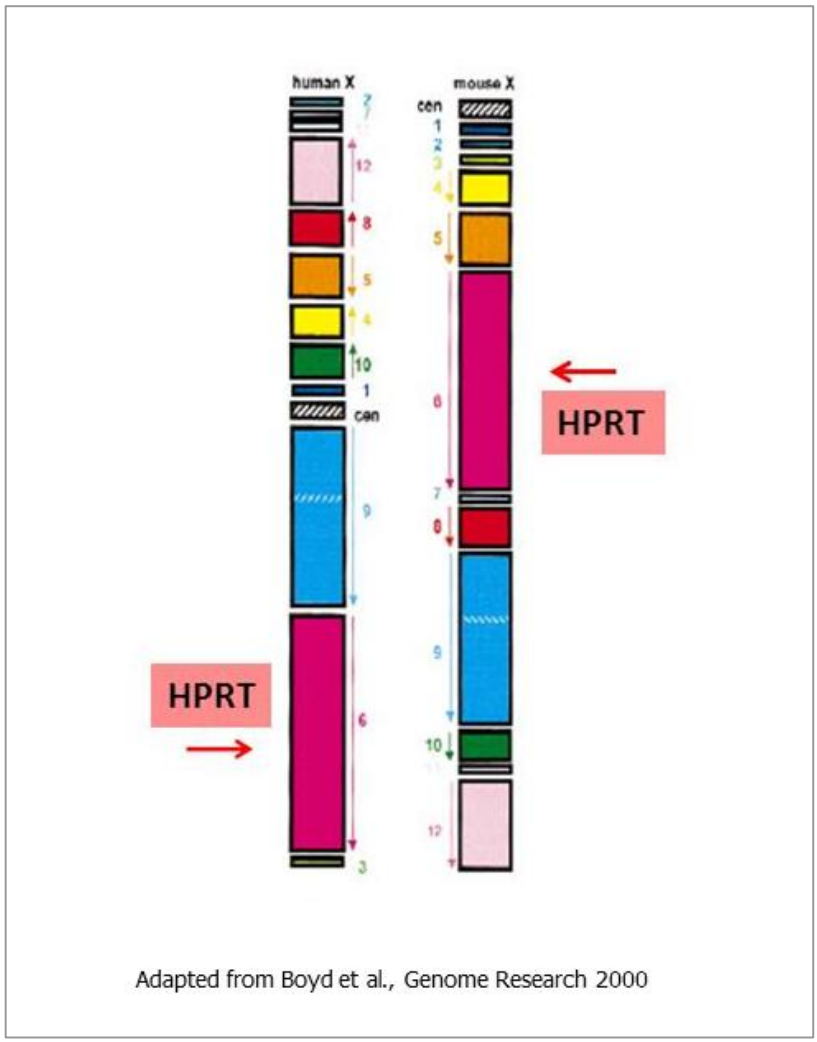

Fig 12 – Comparative phenotype map between human and mouse. Each conserved block is identified by using a different color, and the order of loci inside the block is maintained. A total of twelve regions are recognized as highly conserved region in the comparative human-mouse map. HPRT *locus* (red arrow) is localized in one of the synthenic region.

The HPRT gene is highly conserved both in its position that in the structure. The genomic organization of the human *HPRT* gene and its rodent homologous has been described (Kim et al., 1986; Melton et al., 1984). The mouse HPRT gene covers about 34 kb in length, while the human gene about 40kb, both consisting of 9 exons and 8 introns, and the overall arrangement is similar in both species (Baumstark-Khana et al., 2007) (**Fig 13**). Mouse and human HPRT protein show only eight differences, in that the conservation of the exons and

the length of the derived polypeptides are identical (218 amino-acids) (Baumstark-Khana et al., 2007; Konecki et al., 1982).

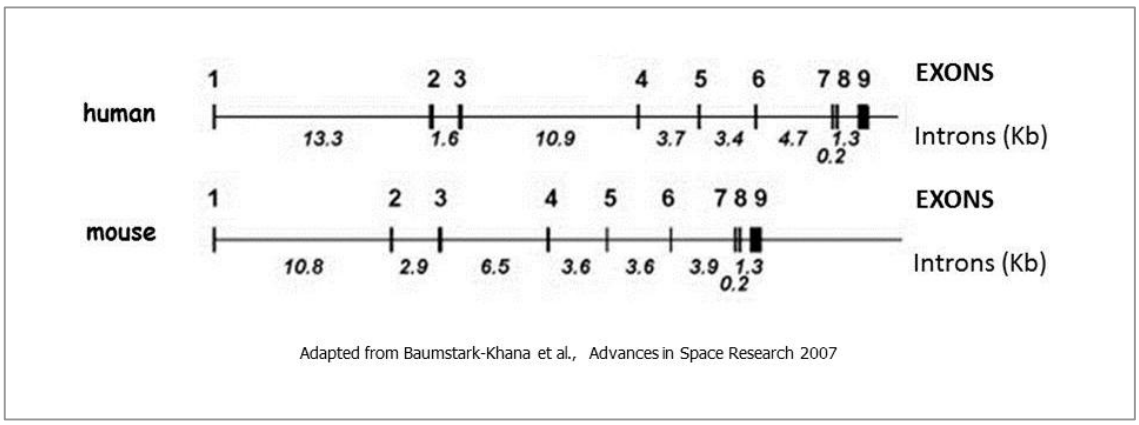

Fig 13 – Gene structure of the human(up) and mouse (down) HPRT *locus*; both consisting of 9 exons and 8 introns. The exons are indicated as black boxes and the locations and sizes of the introns of the gene (in kb) are indicated.

The structural regulatory features are very similar to some of the regulatory signals common to a class of constitutive expressed "housekeeping" genes; in fact the protein is expressed in all tissues in different percentage, but generally at low levels (Kim et al., 1986). Numerous mutations in the protein have been identified, both in patients with LN syndrome and in patients with LN variants, among which the most common are point mutations and deletions (**Tab 2**).

Mutation Category	LND	LNV	Other	Total
Deletion	145	5	7	157
Duplication	37	3	0	40
Other	23	12	1	36
Point mutation	254	120	7	381
TOTAL	459	140	15	614

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Tab 2 - List of the most common mutations in patients suffering from LN and LN variants.

1.4.2. HPRT FUNCTIONS

HPRT gene has been detected in all somatic tissues at low levels (0.005-0.01% of total mRNA), and has showed significantly higher levels in the central nervous system (0.02-0.04%). The enzyme is involved in the salvage pathway of purine (Stout and Caskey, 1985). The synthesis of purines (adenine and guanine) and pyrimidines (timine and cytosine) in the life cycle of a cell is a critical step, since these molecules are the basis of the structure of nucleic acids, and are also involved as coenzymes in metabolic reactions. Most organisms are able to synthesize *de novo* both purines and pyrimidines, but when endogenous purines and pyrimidines are available, the "salvage pathway" to obtain new nucleosides is preferred. It was observed that almost 90% of free purines in humans are recycled, thus preserving as much as possible the energy of the cell. In fact, the classical *de novo* synthesis pathway is much expensive for the cell, since several enzymatic steps need to be performed to obtain inosinate (IMP) from ribose-5-P. Using the salvage pathway, instead, is possible to reuse the bases that are released constantly from the metabolic degradation of the nucleotides in the cell. Guanine and hypoxanthine (the deamination product of adenine) are recovered through its unique transferase reaction catalyzed by HPRT protein. Hypoxantine is modified to IMP that is able to give rise, according two different pathways, adenilate (AMP) e guanilate (GMP). On the other hand, guanine can be converted directly to GMP (Torres and Puig,

2007). In both cases, phosphoribosyl-pyrophosphate (PRPP) molecule has to be used as co-substrate (**Fig 14**).

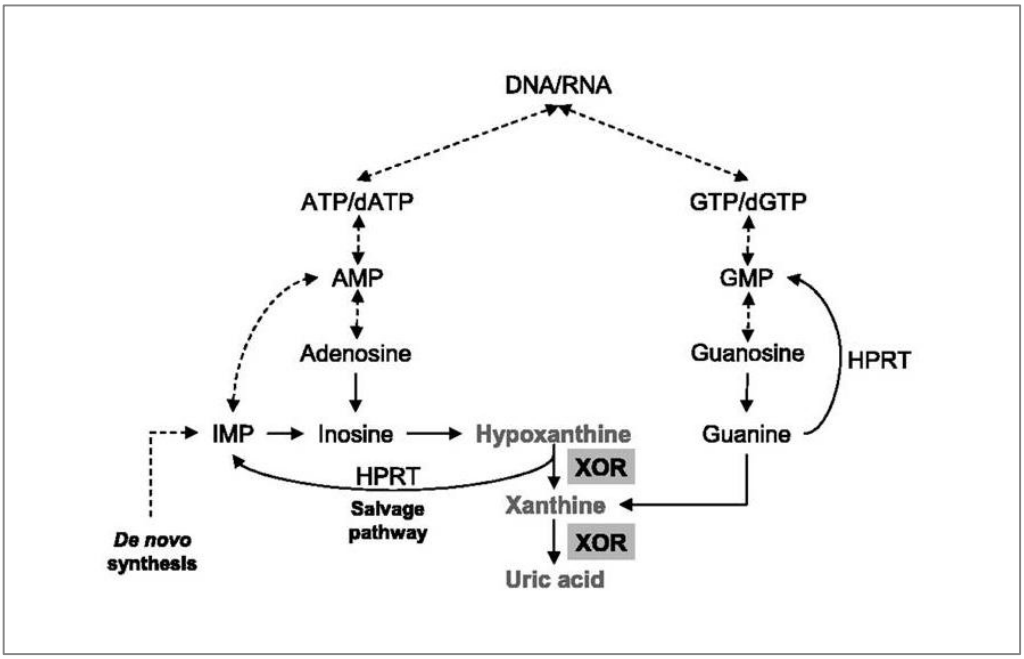

Fig 14 – A simplified overview of the pathways of purine salvage pathway. HPRT enzyme catalyzes the recovery of hypoxanthine and guanine to form respectively IMP and GMP. On the other hand, an accumulation of hypoxanthine and guanine causes the over-production of uric acid.

When the HPRT enzyme is not available, however, an accumulation of precursors occurs, causing over-production of xanthine, whose oxidation produces uric acid. In the brain the purine salvage pathway catalyzed by HPRT is particularly important, for this reason much of the damage that can be observed in patients with LN syndrome are dependent on the nervous system.

HPRT enzyme functionality *in vitro* can be tested using the HAT selection medium. HAT selective medium (hypoxanthine-aminopterin-thymidine medium) was developed for the first time by Littlefield in 1964, and is a selective medium for mammalian cell culture, composed by the combination of aminopterin, a drug that acts as a folate metabolism inhibitor by inhibiting dihydrofolate

reductase, and hypoxanthine and thymidine, which are intermediates in DNA synthesis (Bartal et al., 1987; Ege, 1984). Aminopterin blocks the DNA *de novo* pathway, but at the same time hypoxanthine and thymidine provide cells with intermediates for the activation of the "salvage pathway". In this way it is possible to select HAT resistant cells that do not carry mutations in the HPRT gene, as they can use the salvage pathway in the normal cell duplication. Conversely, HPRT negative cells can be selected in 6-thioguanine (6-TG). 6-TG belongs to the thiopurine family of drugs which are examples of antimetabolites. It is a purine analogue of the base guanine; cells lacking HPRT are resistant to 6-thioguanine (6-TG) and 8-azaguanine. In this way can be selected only the cell population carrying the HPRT gene deletion.

1.4.3. THERAPY

LN syndrome does not have efficient care yet. The production of uric acid using allopurinol can be controlled; this compound blocks the synthesis route, although it has not produced any improvement in behavior and neurological disorders. In addition, the lack of precise knowledge of the dysfunctions at the neurological level has precluded the development of optimal treatment. On the other hand, about the behavioral manifestations, it is possible to use physical restraints and pharmaceutical treatments (Torres et al., 2007). Many research groups have attempted to find a cure for this disease; LN syndrome, being a hereditary genetic disease, has often been the field of gene therapy approaches. Unfortunately, to date no clinically relevant results have been obtained there (McCarthy, 2004).

2. AIM OF THE PROJECT

Several approaches to the correction of genetic defects have been investigated in the last two decades, including those based on viral vectors. Although viral vectors are a powerful instrument for gene therapy and are being used in human trials, they present drawbacks such as risk of insertional mutagenesis and limited insert size. Homologous recombination techniques, which has long been applied to mouse stem cell engineering, have recently been used in combination with novel tools exploiting the activity of nucleases coupled to targeting methods such those based on zinc fingers, TALE and CRISPR, which allow targeting to specific DNA sequences to a great extent. However some genetic defects, and in particular genomic abnormalities such as large deletions, are not suitable to correction with these tools. Here I suggest that large structural abnormalities of the X chromosome can be treated by chromosome transfer mediated by microcells (MMCT). The rationale underlying my work was the fact that by MMCT all the sequences and structures needed for proper expression of the affected gene(s) are transferred into the defective cell. Furthermore, additional X chromosomes are usually silenced and cells with more than two X chromosomes are essentially normal, as shown by XXX trisomic human organisms.

Hence, the aims of the present work are:

1. To define conditions for the MMCT of entire murine X chromosomes into embryonic stem cells

1.1 generation of the donor cell line containing a normal X chromosome
1.2 choice of the recipient ESC line with an X-linked genetic defect
1.3 definition of the best experimental conditions for MMCT

2. To analyze the clones containing a single additional X chromosome

2.1 demonstration of the correction of the genetic defect (HPRT negative, Lesch-Nyhan syndrome) in selected clones

2.2 analysis of X chromosome linked SNP to confirm chromosome transfer

2.3 analysis of the genome stability

2.4 analysis of the pluripotency

3. EXPERIMENTAL PROCEDURES

3.1. CELL CULTURES

HM1 is a mouse embryonic stem cell line (mES) from 129/Ola mouse strain(Hooper et al., 1987; Magin et al., 1992) lacking the HPRT enzyme. This defect prevents its growth in the selective HAT (hypoxanthine 100 uM, aminopterin 0.4 ug/ml and thymidine 16 uM) medium (SIGMA). The embryonic stem cell line was bought from Open Biosystem. The HM1 cell line and the derivative hybrid clones were cultured in 7% CO2 at 37°C on mitomycin-C (SIGMA) treated MEF feeder layer in standard ESC media.

The A9 mouse fibroblast line was derived by Littlefield (Littlefield et al. 1964) from the L cell strain by selection for resistance to 8-azaguanine, since it also lacks the enzyme hypoxanthine-guaninephosphoribosyltransferase (HPRT). For the same reason A9 cells do not grow in HAT selective medium.

MEFs were obtained by mincing and dissociating 13.5 days post coitum (dpc) CD-1 embryos (Charles River Laboratories) by Trypsin-Versene (Lonza); primary cultures were maintained in DMEM medium (Lonza) supplemented with 10% fetal calf serum (Lonza) and incubated at 37°C with 5% CO_2.

C12 and B1 are somatic hybrids obtained by cell fusion between A9 cells and MEF cells. The cell lines are maintained by standard culture procedures in DMEM medium (Lonza), supplemented with 10% fetal calf serum (Lonza) and HAT selective medium (SIGMA), and incubated at 37°C with 5% CO_2.

3.2. WHOLE CELL FUSION AND COLONY ISOLATION

MEF cells and mouse A9 cells were fused according to the subsequent protocol. A total suspension of $2x10^6$ MEF and A9 cells (1:1) were mixed together. After centrifugation (160g), 1 ml of a pre-warmed solution of 50% polyethylene glycol (PEG) (Roche) was poured onto the cell pellet over 1 min and mixed for two minutes. 10 ml of fresh complete medium were then gradually added to the cell suspension over 10 min; the diluted suspension was distributed in three 100 mm Petri dishes and incubated at 37°C with 5% CO_2. The cells were maintained in nonselective medium for 24 hr and then trypsinized and split into ten 100 mm Petri dishes in HAT1x selective medium. Hybrid clones were individually picked, culture expanded and stored; after the analysis, one of them used as donor cells in the microcell mediated chromosome transfer protocol. We have chosen for the subsequent protocol C12 A9/MEF clone; the presence of a normal whole X chromosome was demonstrated by molecular and cytogenetic analysis.

3.3. MICROCELL MEDIATED CHROMOSOME TRANSFER (MMCT) AND COLONY ISOLATION

The C12 A9/MEF hybrid clone, containing a normal X chromosome, was used as source of microcells for the MMCT. C12 cell were seeded and, after reaching 80% of confluence, treated with 0.06 υg/ml of colcemid (KaryoMAX - Life technologies), to induce the formation of micronuclei. After 48 h of incubation the cells were trypsinized, and microcells separated by cytochalasin B digestion

(10 υg/ml, SIGMA) in a Percoll gradient centrifugation. After centrifugation at 16.000g for 70min at 36°C, the isolated microcells were suspended in 12 ml of serum-free DMEM and filtered sequentially through 8 υm and 5 υm polycarbonate filters (Millipore). The purified microcells were collected by centrifugation at 400g for 10min, suspended in 2 ml of serum-free DMEM containing 100 υg/ml of phytohemagglutinin (PHA, SIGMA) and then mixed with an equal amount of mES monodispersed cells. The obtained microcells were fused with HM1 mouse ES unsynchronised cells.

After centrifugation at 160g, 1 ml of a pre-warmed solution of 50% PEG (Roche) was poured onto the cell pellet over 1 min, followed by extensive washing in serum free DMEM. After the cell fusion, cells were seeded on a mytomicine-C arrested feeder layer. The cells were maintained in non-selective medium for 24 hr at 37°C in 7% CO_2, then trypsinized and split into ten 60 mm Petri dishes containing ES medium supplied with HAT 1x.

3.4. CHROMOSOME AND KARYOTYPE ANALYSIS

Chromosome analysis was done both on slide preparations and in suspensions. Briefly, cell cultures were treated with colcemid (KaryoMAX - Life Thecnologies) at a final concentration of 0.1υg/ml for 2h at 37°C, and then mitoses were mechanically removed. After hypotonic treatment with 0.075 M KCl and fixation in methanol:acetic acid (3:1 v/v), the cell suspension was dropped onto a slide and air dried. For slide preparation the procedure was the same. Chromosome counts and karyotype analyses were done on metaphases stained with Vectashield mounting medium with DAPI (Vector laboratories) for G-banding analysis. For the analysis an Olympus BX61 Research Microscope equipped with a cooled CCD camera was used. Images were captured and analyzed with

Applied Imaging Software CytoVision (CytoVision Master System with Karyotyping & FISH).

3.5. FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

Whole mouse X chromosome paint was used as DNA probe for fluorescence in situ hybridization experiments. The probe was obtained labelling X flow-sorted DNA chromosome with degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) using Spectrum Orange-dUTP (Vysis) (Darouich et al., 2012). The labelled probe was suspended in hybridization buffer (50% formamide, 10% dextran sulphate, 1x Denhart's solution, 0.1% SDS, 40 mM Na_2HPO_4 pH 6.8, 2xSSC) containing a 10x excess of Cot1 DNA (Life Technologies); denatured at 80°C for 10 min and pre-annealed at 37° for 30 min. Slides were treated with Pepsin (0.004%) in 0.01 M HCl at 37°C for 2-3 min, washed in PBS, PBS/$MgCl_2$ and subsequently placed in 1% Formaldehyde/1XPBS/MgCl2 for 10 min. Finally, slides were dehydrated through the ethanol series before denaturation in 70% formamide/2xSSC. Hybridization was done overnight at 37°C in a wet chamber. Stringent washings were done in 50% formamide/2xSSC at 42°C. Slides were mounted in Vectashield mounting medium with DAPI (Vector laboratories) and then scored under an Olympus BX61 Research Microscope equipped with a cooled CCD camera. Images were captured and analyzed with Applied Imaging Software CytoVision (CytoVision Master System with Karyotyping & FISH).

3.6. MULTICOLOR- FISH (M-FISH)

The multicolor-FISH (M-FISH) hybridization procedure follows standard FISH protocol with an emphasis on sample preparation. M-FISH was performed using mouse SKY paint probe mixtures (Applied Spectral Imaging, Santa Clara, CA) according to the manufacturer's protocol. The mix probe is shown in the **Tab 3.**

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LABEL	A-RHODAMINE	B-TEXAS RED	C-CY5	D-FITC	E-CY5.5
Abs (nm)	550	596	650	495	675
Em (nm)	570	620	670	525	694

Chromosome	Label
1	ACD
2	CE
3	AE
4	ABD
5	AC
6	BE
7	BD
8	AB
9	BC
10	C

Chromosome	Label
11	E
12	CDE
13	D
14	ACE
15	AD
16	A
17	BCD
18	B
19	DE
X	ABC
Y	ADE

Tab 3 - SkyPaint probe mixture combination (Applied Spectral Imaging, Santa Clara, CA). On the top of the table is shown, for each fluorocrome used, the wavelenght (nm) of emission and absorption spectrum. Each fluorocrome is indicated using a letter. On the bottom of the table is shown the fluorocromes combination specific for each mouse chromosome.

Slide preparation was performed as previously described for the FISH procedure. Hybridization was done at 37°C in a wet chamber for 24-36hr. Stringent washings were done in 0.4XSSC at 72°C for 5min. After the blocking solution two antibodies incubations were done, Cy5 staining reagent and Cy5.5 staining reagent. Finally, slides were mounted in Vectashield mounting medium with DAPI (Vector Laboratories) and then scored under an Olympus BX61 Research Microscope equipped with a cooled CCD camera. Images were

captured and analyzed with Applied Imaging Software CytoVision (CytoVision Master System with mFISH).

3.7. IMMUNOFLUORESCENT ASSAY

For immunostainings, cells were seeded on a mitomycin-C inactivated MEF and, reached the optimal confluence, fixed with 4% PFA for 10min at room temperature, then washed with PBS and permeabilized in 0.3% triton X-100 in PBS for 10min at room temperature.

After blocking incubation using ES-FBS, primary antibodies used were anti Oct4 (Abcam, ab18976), anti Nanog (Novus Biologicals), anti Sox2 (Abcam, ab97959), anti SSEA1 (Cell signaling Technologies, MC480). The primary antibodies incubation was done overnight. Then samples were washed with PBS and incubated with secondary Alexa Fluor®488-coniugated antibodies (Life technologies), diluted 1:2000 in blocking solution. Samples were counterstained with h 4'-6-Diamidino-2-phenylindole (DAPI) 200 µg/ml (Life Technologies) and mounted. Slides were observed using an Olympus BX61 Research Microscope equipped with a cooled CCD camera. Images were captured and analyzed with Applied Imaging Software CytoVision (CytoVision Master System).

3.8. ALKALINE PHOSPHATASE (AP)

For alkaline phosphatase activity tests, ES HM1 or ES hybrid cells were seeded on a mytomicine-C inactivated MEF and used at achievement of 80% of confluence. The tests were conducted using Alkaline Phosphatase kit (SIGMA-ALDRICH) according the manufacturer's recommendations. Slides were scored under an inverted IX53 Microscope.

3.9. REVERSE TRANSCRIPTION-PCR (RT-PCR)

First-strand cDNAs were synthesized directly from ES HM1 and ES "hybrid" cells, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem) according to the protocol provided by the supplier. PCRs were performed using specific primer pairs (**Tab 4**).

	PRIMER FORWARD	PRIMER REVERSE	DIMENSION
OCT4	CTGTAGGGAGGGCTTCGGGCACTT	CTGAGGGCCAGGCAGGAGCACGAG	509
Nanog	CAGAGATCTGCCCACTTATC	CACAGTGTGTGCCAAGACCC	385
SOX-2	GGCAGCTACAGCATGATGCAGGAGC	CTGGTCATGGAGTTGTACTGCAGG	120
GAPDH	TGTCAGCAATGCATCCTGCA	TGGATGCAGGGATGATGTTC	196

Tab 4 - Primer sequences for each pluripotency markers analyzed, and for GAPDH positive control, performed by reverse-transcription PCR. It is shown the nucleotide sequence for both forward and reverse primers, and also the length of bands obtained.

PCR reactions were performed under the following conditions: initial denaturing step for 5min at 94°C, denaturing for 30s at 94°C, annealing temperature for 30s, extension for 30s at 72°C, repeated 30 times; final extension for 10min at 72°C. The PCR products were visualized on 1.5% agarose gel electrophoresis stained with ethidium bromide or gel-red staining.

3.10. TERATOMA FORMATION AND HISTOLOGY

To produce teratomas, $2x10^6$ ES HM1 or ES hybrid cells were inoculated subcutaneously into the flank of six-week-old CD-1 (ICR)-nu mice (Charles River Laboratories). After 2-3 weeks resected teratomas were fixed in 4% PFA and processed for paraffin sectioning, then samples were stained with hematoxylin and eosin (H&E). Histological analyses of the samples were performed by Dr. C. Recordati and Prof. E. Scanziani, Mouse & Animal Pathology Laboratory (MAPLab) Animal Model Systems - FONDAZIONE FILARETE.

3.11. GENOME SNPs

SNPs were analyzed by the polymerase chain reaction (PCR) using specific primers to each genome sequence. We relied on the NCBI database (http://www.ncbi.nlm.nih.gov/projects/SNP/MouseSNP.cgi), for appropriate choice of primers pairs (**Tab 5**).

	PRIMER FORWARD	PRIMER REVERSE	DIMENSION
HPRT WT-T7085F	TTGCAGATTAGCGATGATGAACC		746bp
HPRT Δ-T7697F	CATCTGTAATGGGATCTGGTGCC		628bp
HPRT-T7087R		CCTTACTGTTTTTGCAGAGGACC	
rs3166628	TACATGCACTCTCGAGTGGT	CCAATGGTGCCATTTCTCCA	261bp
rs8251054	TCTGCCTGAGCTCCTTCTTC	AGTTCAATTCCCAGCAACCA	200bp
rs8237198	GGAGTCTAACGCCATTGTCC	TGTACTTCATGGCCTGCTGA	201bp
rs8251080	AGGCATTGACTAGCCAGCAG	AGCCATATTGGCATCCTCAC	295bp

Tab 5 - Primer sequences for each X-chromosome SNP analyzed and for the HPRT *locus*. It is indicated the nucleotide sequence for both forward and reverse primers, and also the dimension (in bp) of band obtained by using PCR assay.

For the analysis of the HPRT *locus* the same reverse primer for both WT and delete allele and a specific forward primer for each allele have been used (McEwan and Melton, 2003); DNA of hybrid clones and parental cells was extracted with GenElute™ Mammalian Genomic DNA Miniprep Kit (SIGMA) according to manufacturer's recommendations.

PCR reactions were performed under the following conditions: initial denaturing step for 5min at 94°C, denaturing for 30s at 94°C, annealing temperature for 30s, extension for 30s at 72°C, repeated 30 times; and final extension step for 10min at 72°C. The PCR products were visualized on 1.5% agarose gel electrophoresis stained with ethidium bromide or gel-Red staining. For sequencing, PCR products were recovered using Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced with specific F primer.

4. RESULTS

4.1. RECIPIENT EMBRYONIC and DONOR CELL LINES

The cell line we selected as recipient cell line, to perform microcell-mediated chromosome transfer (MMCT) protocol, was mouse HM1 embryonic stem cell line. HM1 cell line was purchased from OpenBiosystem, and we have confirmed the pluripotency of cells with the classical tests *in vitro*. The presence of alkaline phosphatase activity in HM1 cell line as first test was proved, as shown in **Fig 15**. Mouse HM1 cell line showed positivity.

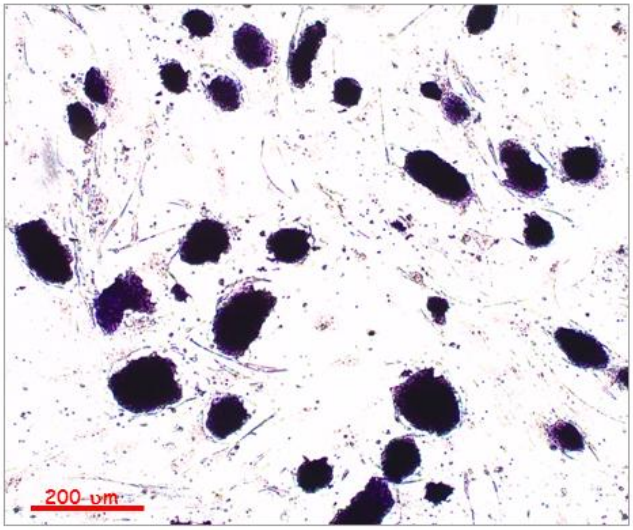


Fig 15 - Alkaline phosphatase activity in HM1 cell line. HM1 cells (positive clones) are seeded on MEF feeder layer (negative cells). 4x magnification, scale bar 200 υm.

To prove chromosome stability *in vitro*, we characterized HM1 cell line with cytogenetic analysis according the Q banding (DAPI staining). We analyzed 30 metaphases, of which 84% had a chromosomal distribution of 2n=40 (**Fig 16**).

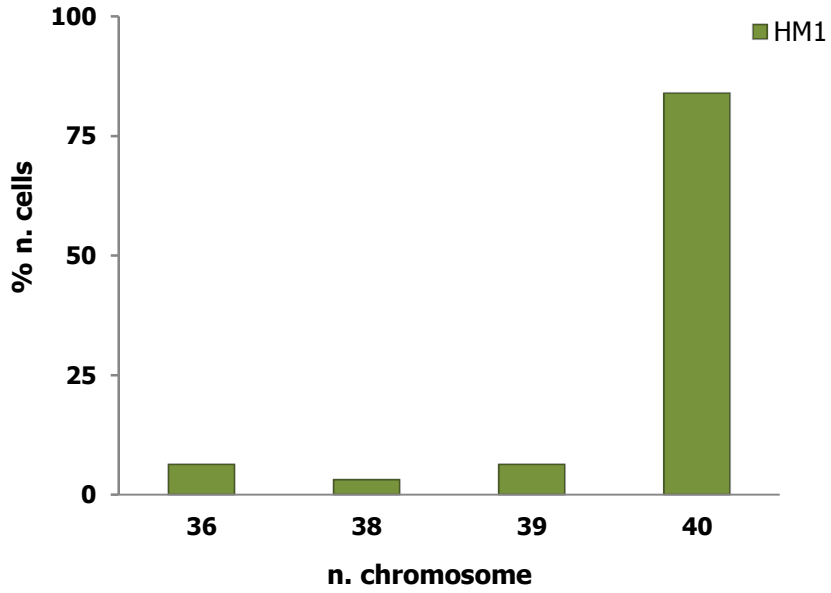


Fig 16 - Chromosomal distribution of the mouse HM1 embryonic stem cell. In the analysis 30 metaphases were tested. The median chromosome distribution was 2n=40.

We investigated also the karyotype, and any chromosomal rearrangements was detected, as shown in **Fig 17** , using a classical Q- banding karyotype.

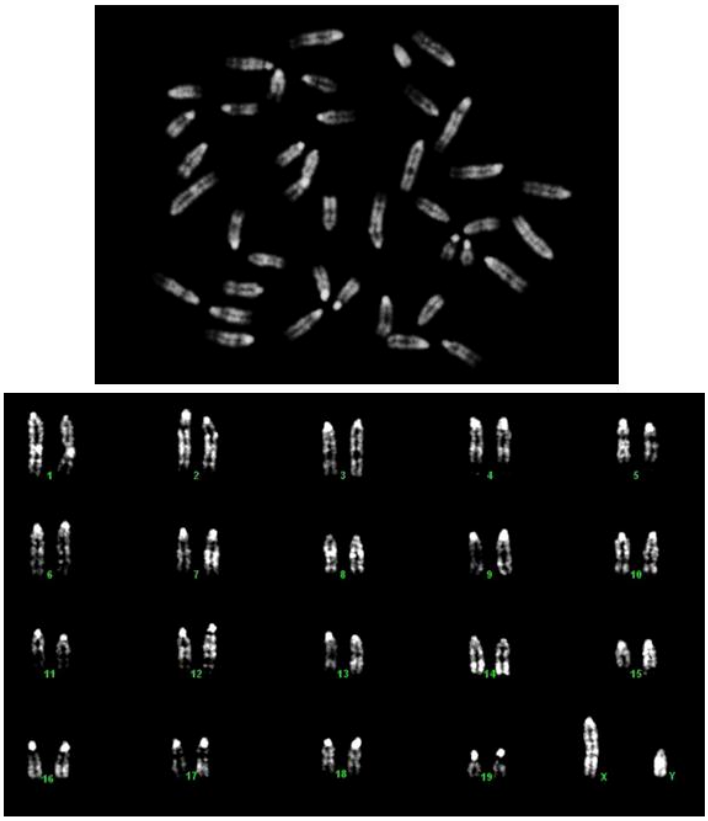


Fig17 - Karyotype analysis of the HM1 embryonic stem cells. The image is representative of a Q-banded karyotype, using DAPI reagent. No rearrangement was detected using the classical DAPI banding.

To show the stemness of the cell line, the classical plurypotency markers Nanog, OCT4 and SOX-2 were analyzed using reverse transcription-PCR (RT-PCR) and immunofluorescence assays. In the RT-PCR assay we used cDNA of E14tg2a cell line as positive control; HM1 parental line showed a gene expression comparable to the positive control (**Fig 18**).

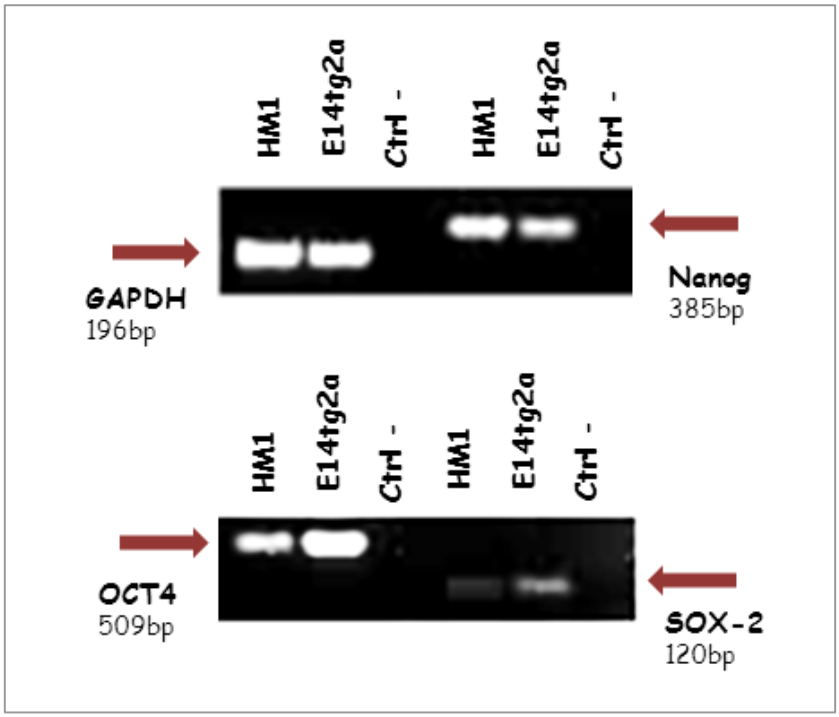


Fig 18 - RT-PCR analysis of pluripotency markers in the HM1 cell line. We tested cDNA of HM1 cell line and cDNA of E14tg2a cell line, used as positive control. Housekeeping GAPDH, Nanog (top), OCT4 and SOX-2 (bottom) cDNAs were analyzed. HM1 cell line showed an expression comparable to the positive control for all markers analyzed. To the left of each marker the size (bp) of the amplified band is indicated.

In the immunofluorescent assay we tested the most common pluripotency markers Nanog, Oct4, SSEA-1 and SOX-2; all markers analyzed were expressed (**Fig 19**).

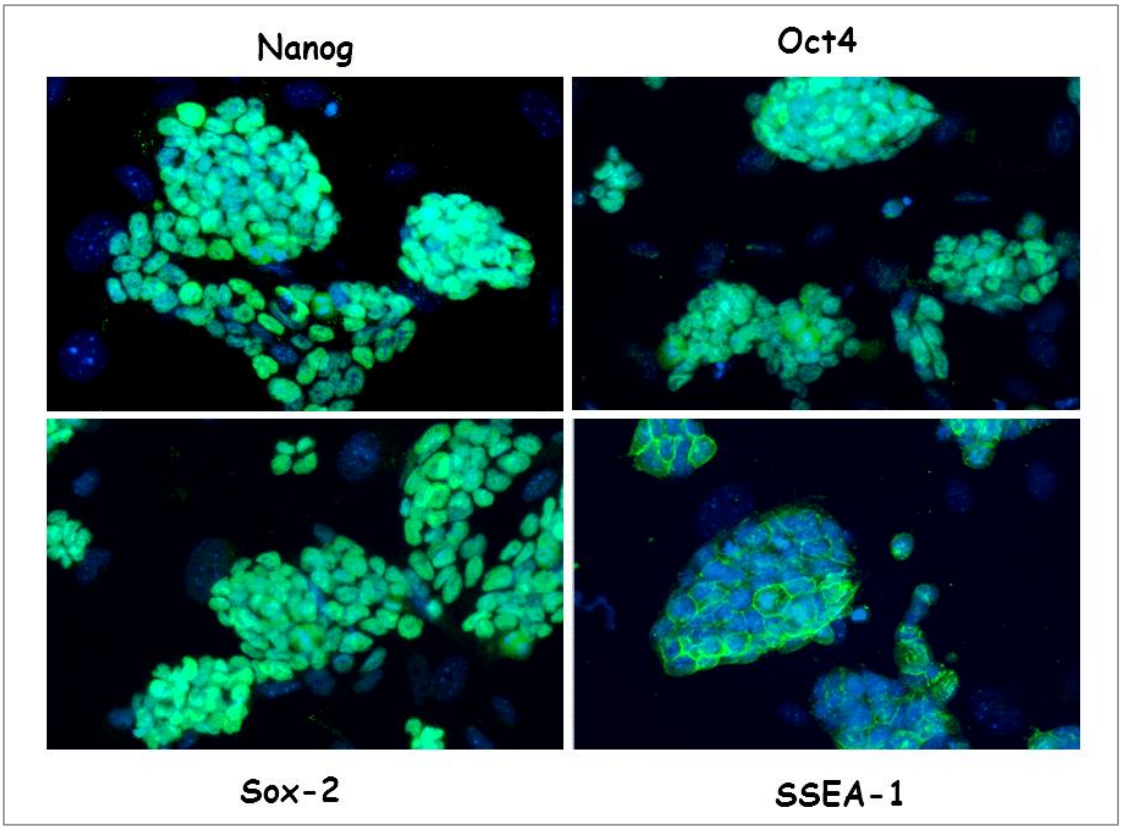


Fig 19 - Immunofluorescence assay of pluripotency markers in the HM1 cell line. Oct4, Nanog, Sox-2 and SSEA-1 markers were tested; in each image are visible clones stained with an anti-Oct4, anti-Nanog, anti-Sox-2 and anti-SSEA-1 antibody (green fluorescent signals), counterstained with DAPI (blue). All markers showed clear positivity.

As X chromosome donor we used an A9/MEF hybrid cell line generated *de novo*. To this aim, we started with the A9 murine fibroblast cell line that is an HPRT mouse defective cell line, which easily micro-nucleates and has previously been used to transfer single chromosomes (Kugoh et al., 1999). The A9 cell line has a modal number of 52 chromosomes; range between 46 to 54 chromosomes, and is a hyperdiploid cell line with 24 to 26 marker chromosomes common to all cells; structurally normal chromosomes 4, 15, 16, 17, 18 and X are absent. We analyzed the A9 cell line to investigate the chromosome pattern in culture. As can be observed in the **Tab 6**, the median chromosome distribution is 56, counting 20 biarmed chromosomes, resulting

from the centromeric fusion of acrocentric chromosomes, 35 telocentric chromosomes and two dot-like structures.

	Ch. BIARMED	Ch. TELOCENTRIC	DOT-LIKE	Ch. TOTAL
A9 cell line	20	35	2	56

Tab 6 - Chromosomal characterization of the A9 cell line in culture. In each metaphase the number of chromosomes was counted, and the chromosome median for each class is shown: biarmed chromosomes, telocentric chromosomes and dot-like structures.

Since this line is defective for the HPRT gene, successful normal X chromosome transfer, bearing a normal HPRT gene, can be selected in HAT medium. To prove that, we maintained A9 cells in HAT selection medium, and the cells died; in addition, FISH experiments with a mouse X chromosome probe confirmed the absence of a normal single X chromosome (**Fig 20**).

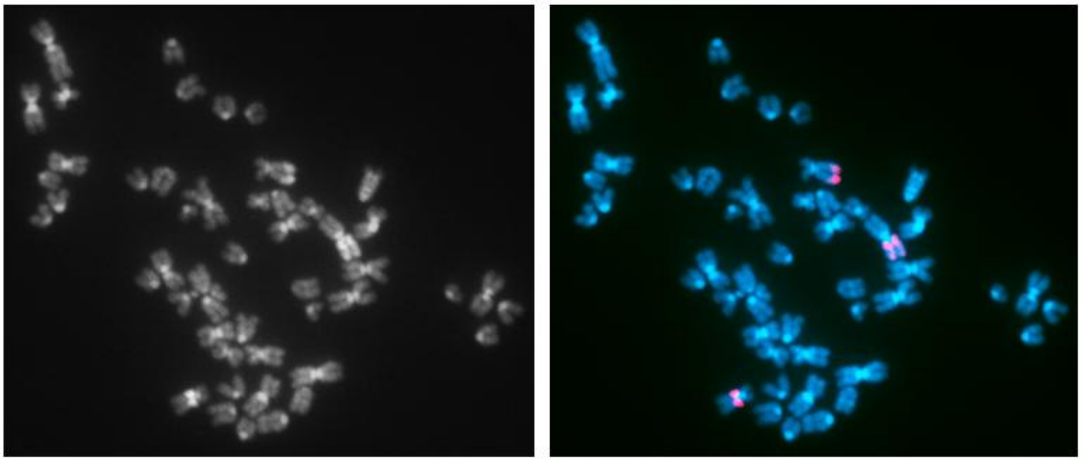


Fig 20 - FISH image of metaphase spreads prepared from the A9 cell line, performed with mouse X chromosome paint labeled with Spectrum Orange (red), and counterstained with DAPI (blue) (merge on the right). DAPI-stained chromosomes were converted into a black and white image (on the left). No whole normal X chromosome can be observed. Magnification 60x

MEF pool was obtained by sacrificing at day 13.5 a healthy female CD1 pregnant mouse and recovering embryos to obtain a primary line of fibroblasts (MEF), which were cultured and collected in a pool of cells. It is important to note that this pool was composed by XX and XY cells, derived from different embryos, each carrying one (male embryos) or two (female embryos) normal X chromosomes.

4.2. GENERATION OF THE A9 HYBRID CELL LINE WITH A NORMAL X CHROMOSOME BY WHOLE CELL FUSION

We performed a whole cell fusion protocol to obtain A9/MEF hybrid clones, able to micro-nucleate, to have a donor cell line carrying a normal mouse X chromosome. A9 cells were fused with MEF pool cells. In the whole-cell fusion protocol, two million MEF and A9 cells in a 1:1 ratio were fused together in suspension using a PEG fusogen (1 minute). The cells were then re-plated using A9 complete medium and the HAT selection was added the following day. Seven clones were obtained, grown separately and always maintained in selective medium, to avoid any possible contamination of whole A9 cells. A chromosomal distribution of every clone has been performed as first analysis. In the **Tab 7** the median chromosome distribution for all clones is shown, classifying them as biarmed chromosomes, telocentric chromosomes and dot-like structures.

CLONE	Ch. BIARMED	Ch. TELOCENTRIC	DOT-LIKE	Ch. TOTAL
C12	22	67	1	90
D2	23	48	2	73
B1	22	52	1	76
C9	18	49	2	67
C11	23	48	2	73
G9	24	45	3	72
A9	20	35	2	56

Tab 7 - Chromosomal characterization of A9/MEF hybrid clones obtained after whole cell fusion. In each metaphase the number of chromosomes was counted, and the chromosome median is shown, classifying them in biarmed chromosomes, telocentric chromosomes and dot-like structures.

To identify among the clones obtained the best ones for our purpose, we performed FISH experiments on chromosome preparations obtained from individual clones, using an X chromosome paint probe. We identified two clones, C12 and B1, on which FISH experiments revealed the presence of at least one normal whole X chromosome. In particular, while in the C12 hybrid clone only one normal X chromosome was identified, in B1 clone two normal X chromosomes were detected. We analyzed 30 metaphases for each FISH experiment, and a representative image per each clone is shown in the **Fig 21**.

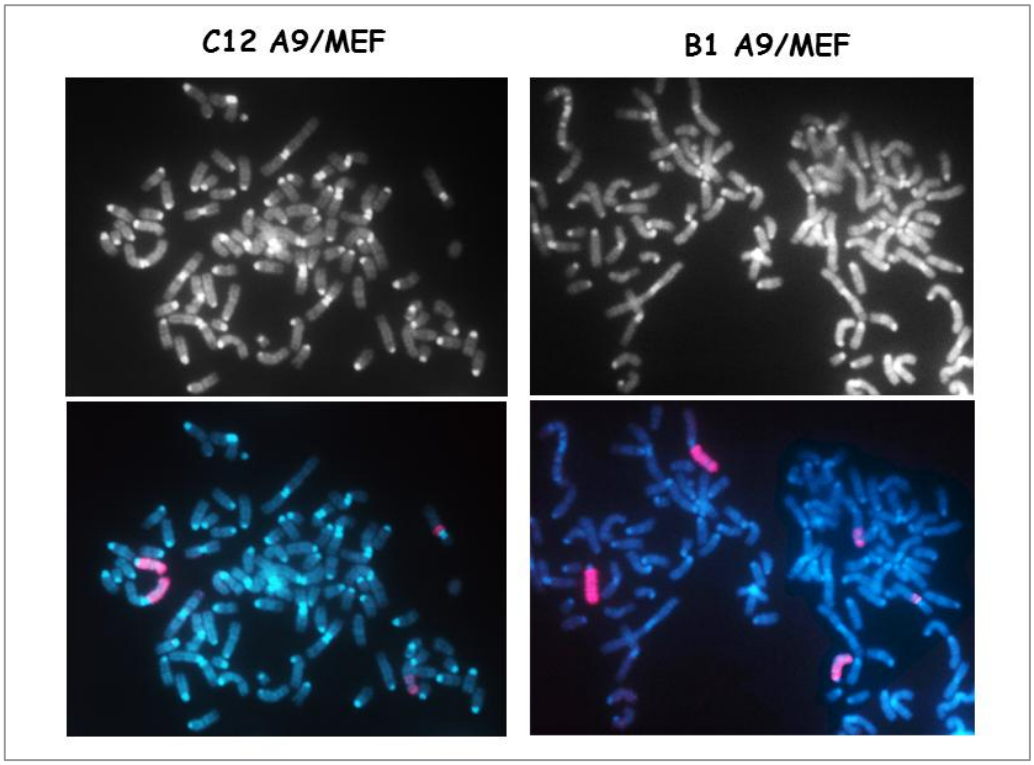


Fig 21 - FISH experiment on metaphase spreads prepared from B1 A9/MEF (on the right) and C12 A9/MEF clones (on the left), performed with a mouse X chromosome paint probe labeled with Spectrum Orange (red) counterstained with DAPI (blue) (merge on the bottom). Every DAPI-stained methaphase was converted into a black and white image (top). In B1 clone two X chromosomes can be visualized, while in clone C12 only one normal chromosome can be detected, beyond those rearranged already present in the starting A9 cell line. Magnification 60X.

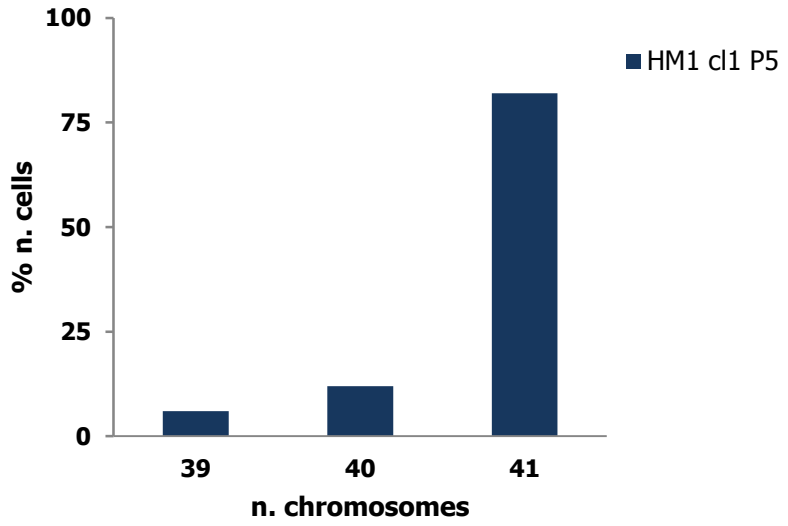
The presence of an active HPRT gene was demonstrated through the survival C12 and B1 clones in HAT selection medium.

4.3. MMCT FUSION

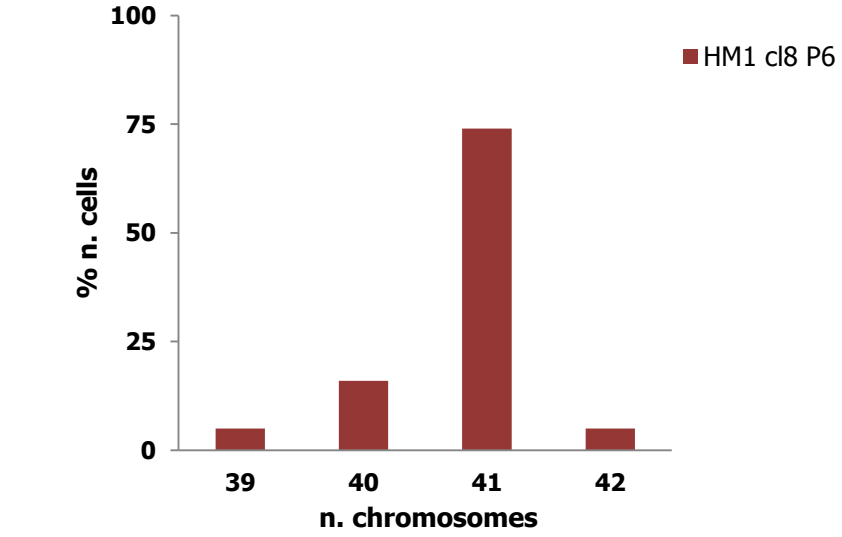
Microcell-mediated chromosome transfer (MMCT) experiment was done according to the method described in Experimental Procedures. The hybrid C12 clone was chosen because it showed a better ability to micronucleate. The C12 clone, reached the 80% of confluence, was treated with 0.06 υg/ml colcemid, to induce the formation of micronuclei. After 48 h incubation, the microcells were isolated by cytochalasin B digestion (10 υg/ml) and Percoll centrifugation ($5*10^7$ microcells were counted). After centrifugation, the isolated microcells were filtered sequentially through 8 υm and 5 υm polycarbonate filters and after this step $1.6*10^7$ single microcells were collected. The purified microcells were suspended in DMEM containing 100 υg/ml of phytohemagglutinin (PHA) and then mixed with an equal amount of parental HM1 monodispersed cells. After centrifugation a pre-warmed solution of 50% PEG (Roche) was poured onto the cell pellet over 1 min, followed by extensive washing in serum free DMEM. The cell suspension was seeded on mitotically inactivated fibroblasts. The day after fusion, HAT 1x selective medium was added and after two weeks two clones were obtained, grown in selection medium and individually expanded in culture for the subsequent analysis.

4.4. GENERATION AND ANALYSIS OF CORRECTED CLONES BY MMCT

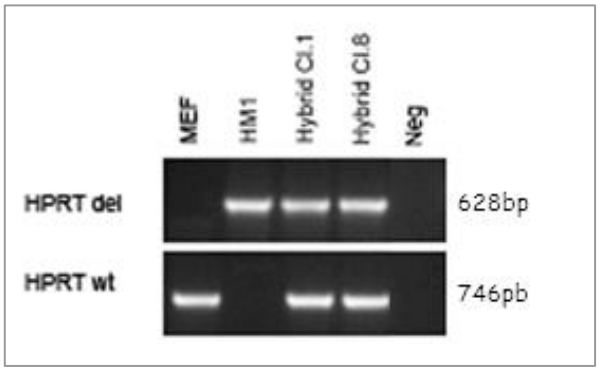
To prove the insertion of a normal X chromosome in the mouse recipient cell line, chromosome distribution of both HAT-resistant clones was performed. We analyzed 30 metaphases for both MMCT clones, and both showed a chromosome distribution of 2n=41 (**Fig 22 A, B**). The HPRT gene PCR amplification (**Fig 22 C**), performed on HM1 cl8 and cl1, showed the presence of both the normal (746 nt band) and the mutated (628 nt band) HPRT allele, confirming that a wild-type copy of the gene from the normal X chromosome has been acquired.



A



B

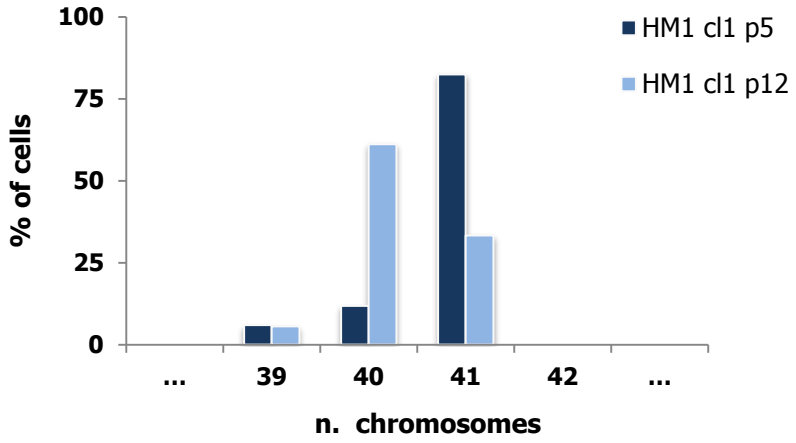


C

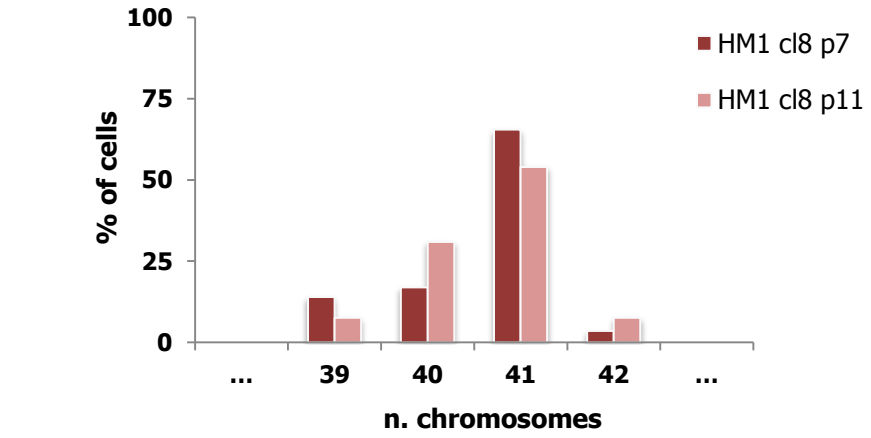
Fig 22 - Chromosomal distribution and genomic PCR analysis performed on HM1 cl8 and cl1. Chromosomal distribution of HM1 cl1 (**A**) and HM1 cl8 (**B**), performed at low passages *in vitro*. Both clones showed a median chromosome distribution of 2n=41. HPRT gene PCR amplification (**C**) showed the presence of both normal and mutated alleles in MMCT clones obtained, due to the transfer of the wild-type copy of gene within the normal X MEF chromosome. In the HM1 parental line only the HPRT mutated allele is present, while in the MEF donor cells only the HPRT wild-type allele is shown.

4.4.1. GENOME STABILITY OF THE OBTAINED CLONES

The two MMCT clones were maintained in culture for three weeks. Whereas HM1 cl1 had a propensity to return to a chromosome distribution of 2n=40 (**Fig 23 A**), the HM1 cl8 showed a more stable pattern, maintaining the chromosomal distribution of 2n=41 (**Fig 23 B**).



A



B

Fig 23 - HM1 cl1 and cl8 chromosome distribution at different passages *in vitro*. HM1 cl1 (**A**) showed an unstable chromosome pattern after several *in vitro* passages, changing from 2n=41 to 2n=40. HM1 cl8 (**B**) showed a more stable pattern, maintaining the chromosomal distribution of 2n=41.

We therefore decided to use HM1 hybrid cl8 because it seemed more stable, and we continued the analysis. To follow the trend of the clone *in vitro* we repeated distribution chromosome analysis at different steps, and even at increased passages the clone showed a stable chromosome distribution. We have also tested the chromosomal stability *in vitro* after adding and removing the HAT selective medium, and also in this case the clone kept its original distribution of 2n= 41 chromosomes (**Fig 24**).

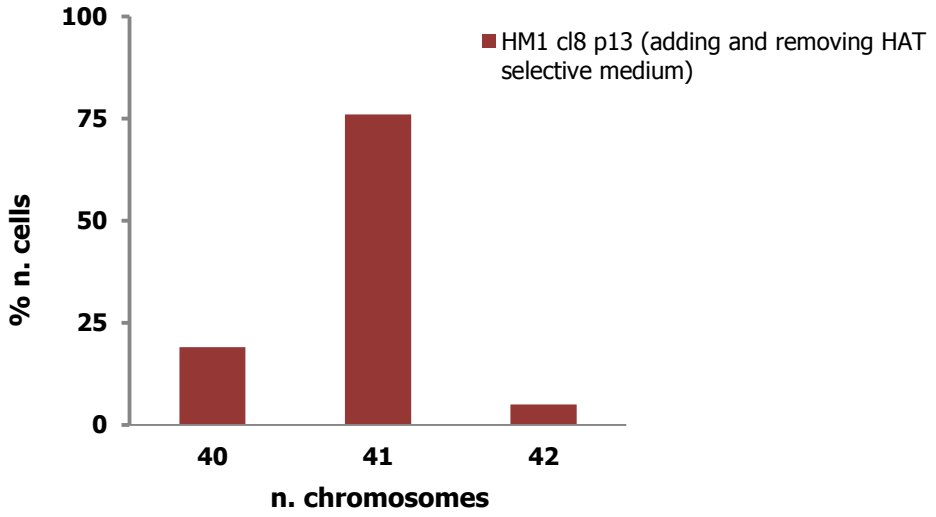


Fig 24 - HM1 cl8 chromosome distribution after adding and removing HAT selective medium. The clone has been cultured for seven passages in HAT selective medium, then for three passages without selective medium and finally the HAT medium was re-added in culture. The median chromosome distribution remained stable at 2n= 41.

4.4.2. HM1 CL8 CHARACTERIZATION

4.4.2.1. Molecular analysis

To further demonstrate the MEF X chromosome transfer into the mouse HM1 hybrid cl8, SNP analysis of X chromosome was performed. We have used the SNPs mouse database of NCBI (http://www.ncbi.nlm.nih.gov/projects/SNP/MouseSNP.cgi), for the X chromosome, where the C57 strain is reported. Since the HM1 parental cell line derived from 129/Ola strain, while the MEF donor cell line derived from the CD1 strain, we chose some informative SNPs for our purpose, that are those in which the nucleotide sequence is different in the two strains. In this way it is possible to distinguish the donor X from the HM1 parental chromosome. We have tested several SNPs by PCR analysis of HM1 and MEF genome, and four informative SNPs were found; then, PCR amplification and sequence analysis were performed. As shown in the **Tab 8**, analyzing

HM1 cl8 genome, both the endogenous and exogenous SNPs X chromosomes were present.

Position	Ch X 50372156	Ch X 70963328		Ch X 131081377	Ch X 131108926		Ch X 131126015
SNP	HPRT Δ/WT	rs3166628		rs8251080	rs8251054		rs8237198
HM1 129/Ola strain	Δ	C	T	G	C	T	A	A
MEF CD1 strain	WT	A	A	C	C/G	C/T	A/G	A/G
HM1 CL8	Δ/WT	A/C	A/T	C/G	C/G	C/T	A/G	A/G

Tab 8 - Informative SNPs of the X chromosome. In addition to the HPRT gene, four informative SNPs were found as different between the 129/Ola and CD1 parental strains used. For each SNPs is indicated the position (top) and the specific nucleotide polymorphism of HM1 129/Ola, MEF and HM1 cl8. As the hybrid HM1 cl8 has two X chromosomes, each derived from the two original strains, it shows the presence of both nucleotides. In rs3166628 and rs8251054 SNPs, in addition to the published in the database, we found two additional polymorphisms within the amplified region in the first region, and one in the second amplified genomic region.

4.4.2.2. Cytogenetic analysis

Analyzing the clone 8 karyotype, no obvious rearrangements was identifiable by the classic Q- banding DAPI, as shown in **Fig 25**. The karyotype of 20 metaphases was analyzed, and the analysis pointed out that the all autosomes and sex chromosomes were normal in number and structure and that there were no rearrangement or aneuploidy detected by the classic DAPI banding. Compared to the parental HM1 cell line, only a normal X chromosome was inserted.

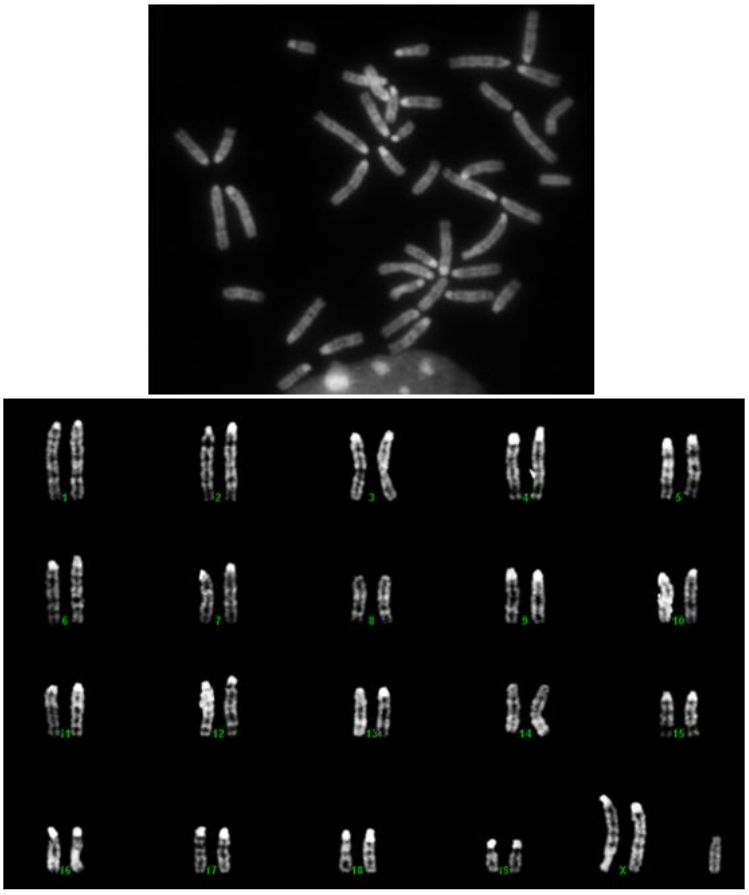


Fig 25 - HM1 cl8 karyotype. The image is representative of Q-banded karyotype, obtaining using DAPI staining. No detectable rearrangements or aneuploidy are visible.

To avoid the possibility of rearrangements involving X chromosome, and to confirm the presence of two whole and normal X chromosomes in HM1 cl8, FISH experiments were carried out, using mouse chromosome X paint probes. 30 metaphases were analyzed, and as shown in **Fig 26**, FISH analysis detected in hybrid cl8 signals originating only from two structurally normal X chromosomes, demonstrating that the MMCT procedure did not cause any rearrangements of the original and transferred X chromosome.

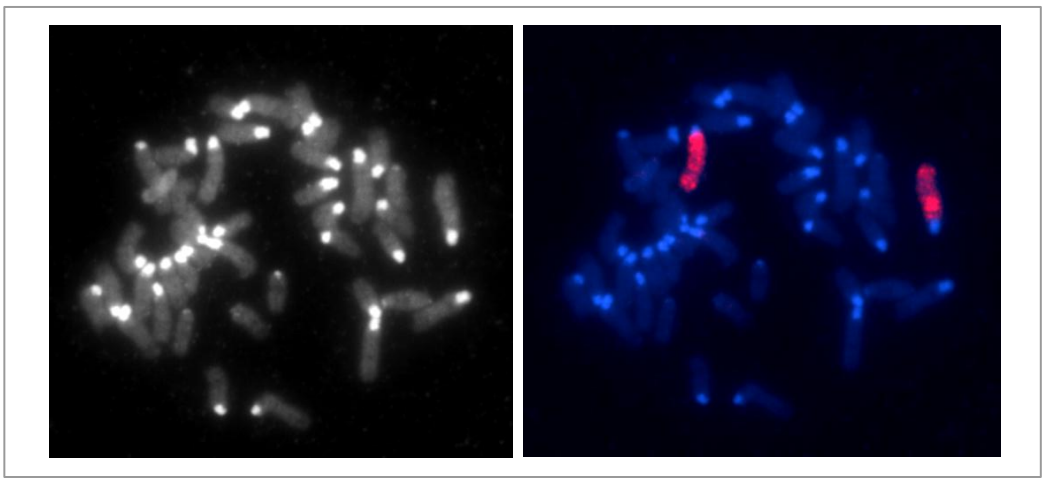


Fig 26 - FISH experiment on metaphase spreads prepared from HM1 cl8 p5, performed with mouse X chromosome paint labeled with Spectrum Orange (red) counterstained with DAPI (blue)(merge on the right). Every DAPI-stained methaphase was converted into a black and white image (on the left). Two normal X chromosomes can be visualized. Magnification 60x.

To confirm the absence of rearrangements or aneuploidy both in the autosomes and the sex chromosomes after *in vitro* manipulation experiments, a multicolor-FISH (M-FISH) was performed. M-FISH uses a pool of whole-chromosome painting probes with different fluorochromes combinations that result in specific color patterns for each chromosome. M-FISH experiments were performed by using Applied Spectral Image (ASI) probes as described in Experimental Procedure. Again, no chromosomal rearrangements were identified (**Fig 27**). In conclusion, HM1 cl8 can be considered to have acquired an additional, HPRT positive, chromosome without any rearrangement of the original chromosome pattern.

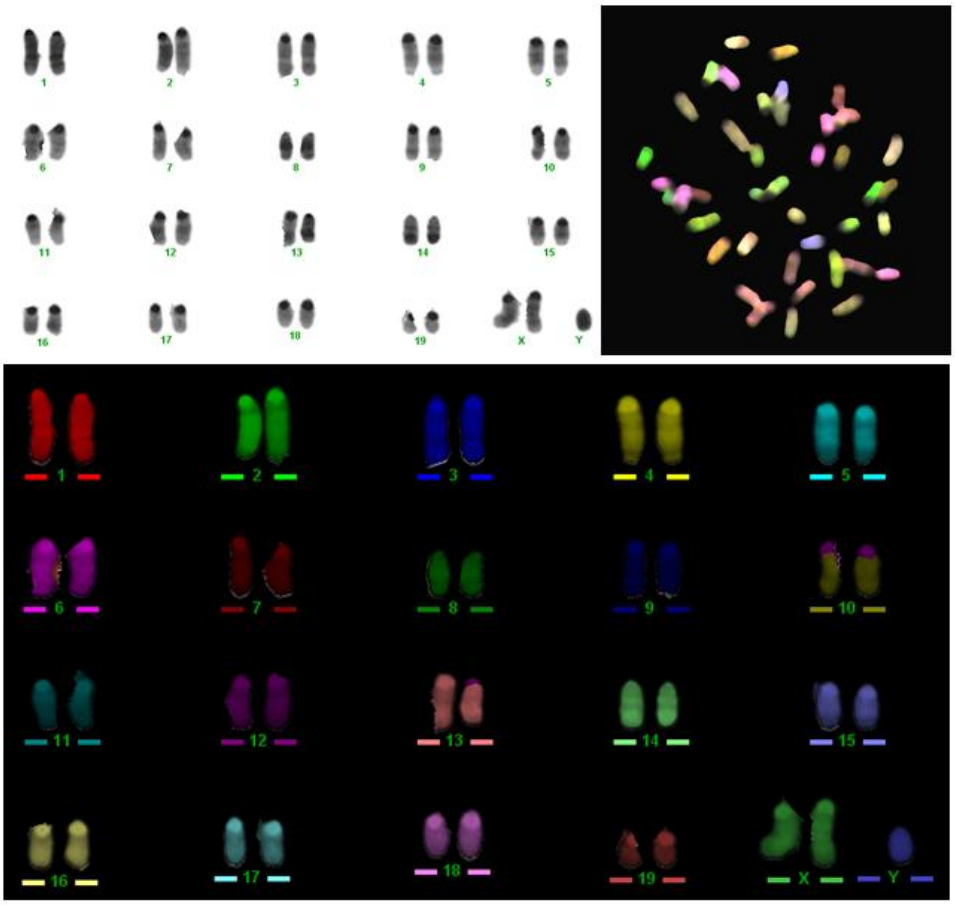


Fig 27 - M-FISH analysis on metaphase spreads prepared from HM1 cl8, performed with Applied Spectral Image kit probes (ASI). The karyotype is shown in inverted DAPI-banded (left), and in the pseudocolor image (on the bottom). The hybridization signal is visible on the upper part of panel (right). Analyzing karyotype, no rearrangement can be noted.

4.4.2.3. Stemness

To analyze the pluripotency of the hybrid cl8 obtained, alkaline phosphatase activity was tested (**Fig 28**); as expected the clone retained the enzymatic activity as well as the parental HM1 cell line.

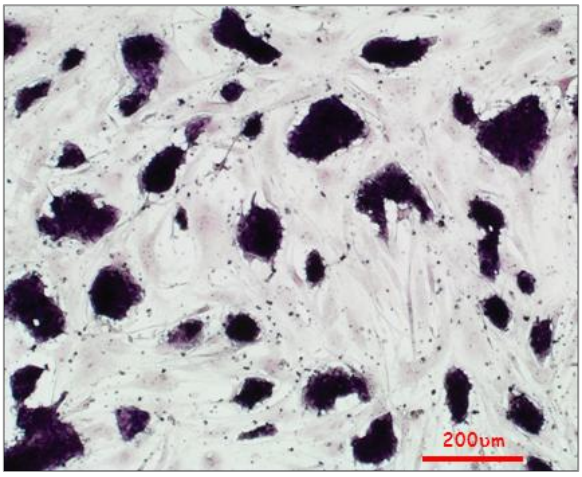


Fig 28 - Alkaline phosphatase activity in HM1 cl8 hybrid line. Cl8 cells (positive clones) are seeded on MEF feeder layer (negative). The hybrid clone retained the enzymatic activity as well as the parental HM1 cell line. Magnification 4x, scale bar 200υm

In addition, immunofluorescence assays for the classical pluripotency markers Oct4, Nanog, Sox-2 and SSEA-1 was performed; the clone showed positivity for all markers examined (**Fig 29**).

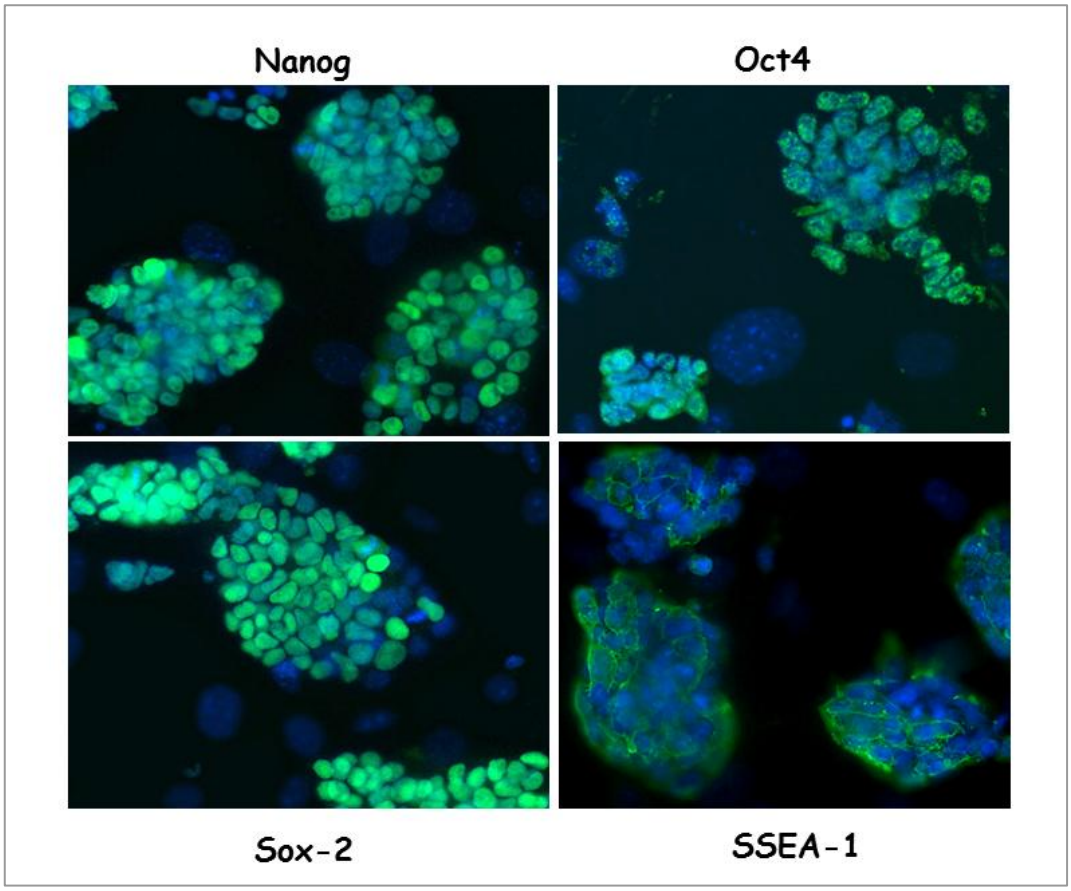


Fig 29 - Immunofluorescence assay of hybrid HM1 cl8. Oct4, Nanog, Sox-2 and SSEA-1 markers were tested; in each image are visible clones stained with an anti-Oct4, anti-Nanog, anti-Sox-2 and anti-SSEA-1 antibody (fluorescent green signals), counterstained with DAPI (blue). All markers showed clear positivity.

To complete the analysis of pluripotency *in vitro*, the presence of Nanog, OCT4 and Sox2 pluripotency markers was also tested by reverse-transcription-PCR. As shown in **Fig 30**, all markers were expressed. GAPDH was used as cDNA positive control in that is a housekeeping gene; in the assay we used cDNA of HM1 cell line as positive control. HM1 cl8 expression was comparable to HM1 parental line.

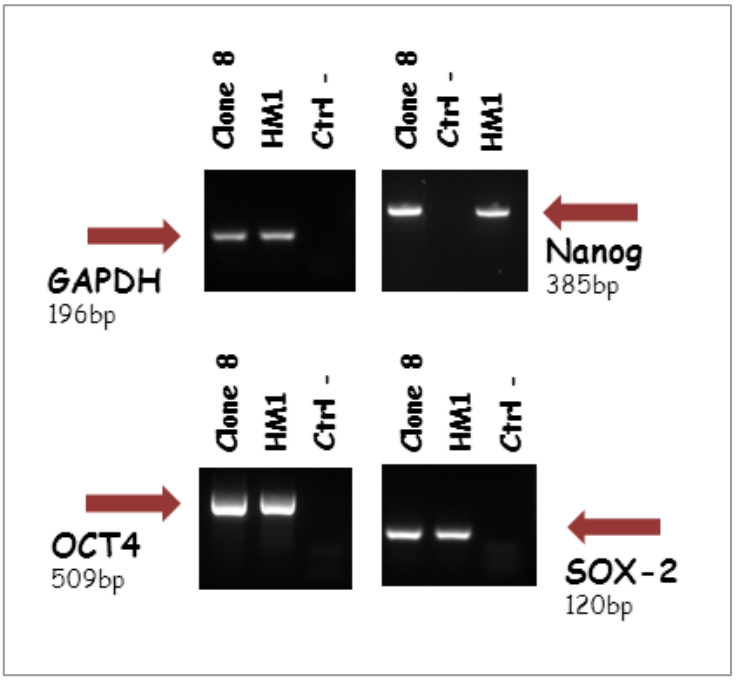


Fig 30 - RT-PCR analysis of pluripotency markers in the HM1 cl8 cells. We tested cDNA of HM1 cl8 cell line and cDNA of HM1 cell line, used as positive control. Housekeeping GAPDH, Nanog (top), OCT4 and SOX2 (bottom) cDNAs were analyzed. HM1 cl8 showed an expression comparable to the positive control for all markers analyzed. To the left of each marker the size (bp) of the amplified band is indicated.

Finally, to test the capability of HM1 cl8 cells to differentiate *in vivo*, about two millions of cells were inoculated into the flanks of six-week-old CD-1-nu-nu mice, to test the ability to form teratoma structure. After three/four weeks mice were sacrificed, and the resected teratomas were fixed in 4% PFA and processed for paraffin sectioning and staining. According the analysis performed, the hybrid cl8 showed the capability to generate keratinized squamous epithelium (ectoderm), skeletal muscle (mesoderm) and ciliated epithelium goblets (endoderm). Therefore hybrid cl8 cells were able to generate all three germ layers as the original plurypotency stem cells (**Fig 31**).

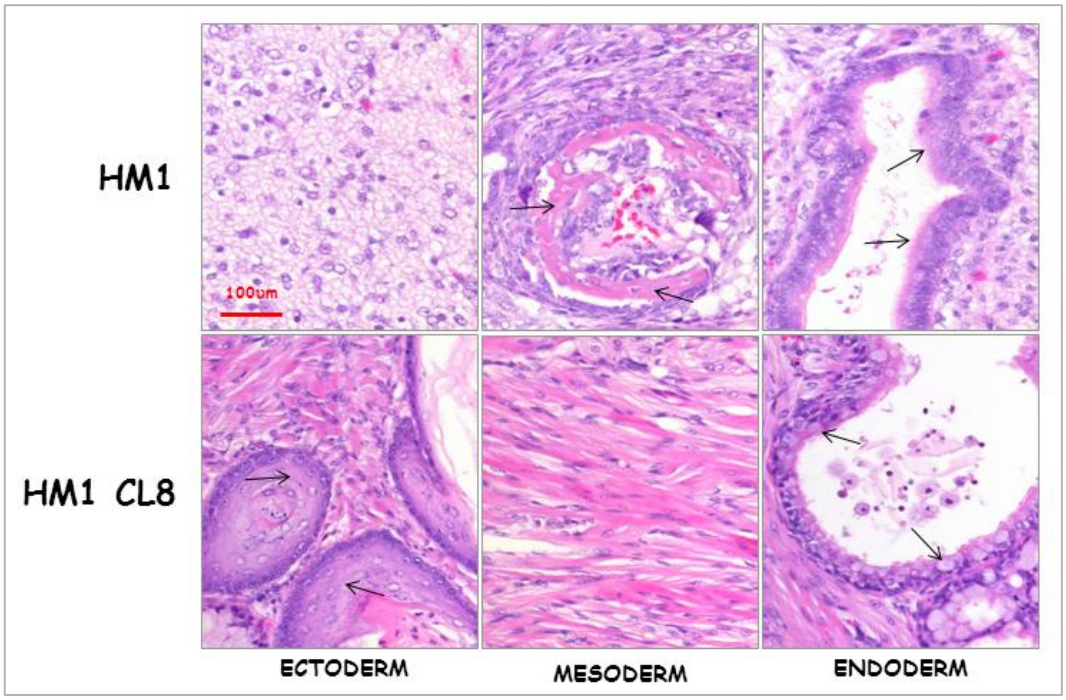


Fig 31 - Histological analysis of teratoma formed three/four weeks after subcutaneous injection in immunocompromised six-week-old CD-1-nu-nu mice. The hybrid cl8 cells were able to generate all three germ layers as the HM1 parental pluripotent stem cells. The HM1 images represent nervous tissue (ectoderm), bone tissue (mesoderm) and ciliated epithelium (endoderm) from the left to the right. The HM1 cl8 images show keratinized squamous epithelium (ectoderm), skeletal muscle (mesoderm) and ciliated epithelium goblets (endoderm). Black arrows indicate the derived tissues (Hematoxylin and Eosin staining, 400x). Scale bar= 100υm.

In conclusion, we were able to produce, by MMCT, ESC clones with an additional normal X chromosome, which corrects the genetic defect in the HPRT gene, as shown by the ability of the clones to grow in HAT medium. Some of these clones were karyotypically normal and maintain their pluripotency in spite of all the manipulation steps described above.

5. DISCUSSION

We have demonstrated that a single normal mouse X chromosome can be transferred from a donor cell line to a recipient cell line by using microcell-mediated chromosome transfer (MMCT) protocol, with the final aim to cure inherited diseases caused by large gene deletions or rearrangements involving X chromosome. In fact, the classical gene therapy approaches do not allow the cure of diseases caused by large chromosomal rearrangements, due to the low efficiency of the available methods, or to the size limits of the vector used to convey therapeutic molecules. In our project we wanted to show that by using MMCT all the sequences and structures needed for proper expression of the affected gene(s) are transferred into the defective cell allowing the restoration of a complete large genomic region.

To do that, we have therefore chosen as proof of principle of our project a X-linked genetic disease, Lesch Nyhan syndrome (LN), which has been described to be due to both point mutations and large deletions involving the HPRT gene. In LN patients, the loss of the enzyme causes the impairment of the purine metabolism pathway, causing an over-production of uric acid. It is important to emphasize that so far LN disease does not has an effective cure yet, but only palliative treatments. Our goal was to demonstrate the efficiency of our gene therapy approach in this disease model, for which viable therapies do not yet exist. So, we chose the HM1 embryonic cell line that works as an excellent mouse model of the LN disease for *in vitro* manipulation; in fact the recipient HM1 stem cell line, a male line with normal chromosome content, carries a deletion in the HPRT gene. Furthermore, this cell line gave us the opportunity to use a very efficient selection system *in vitro*, the HAT selection medium.

The HM1 cell line has been isolated directly from HPRT-deficient strain 129 mice, and is characterized by a deletion of the HPRT gene, which extends from at least 10 kilobases 5' to the promoter up to the second intron of the genomic sequence (Doetschman et al., 1987; Thompson et al., 1989).

To perform our project, our first goal was to obtain a donor cell line, carrying at least one normal X chromosome, capable to efficiently form micronuclei. For the hybrid donor cell line generation we started from the A9 cell line. We have chosen this cell line because it was very efficient in micro-nucleation and has already been used by others to transfer single chromosomes (Kugoh et al., 1999). The A9 cell line did not contain any normal X chromosome, as we have highlighted by FISH experiments using X mouse chromosome paint probe, confirming the data already present in literature. In addition, A9 is HPRT defective.

So as a first step we performed a whole cells fusion experiment between the A9 cell line and fetal fibroblasts (MEF) with PEG as fusogen agent. Our goal was to obtain a mono-chromosomic hybrid clone that presented an additional normal, MEF-derived X chromosome, together with the A9 chromosomes parental line. Since the acquired normal X chromosome provided the A9 cell line with a functional HPRT gene, the positive clones could be selected in HAT selective medium.

Using this protocol, we obtained several clones. Our attention focused on two A9-like HAT resistant clones, C12 and B1, both having at least one whole X chromosome, as was demonstrated by the FISH experiments performed with the mouse X chromosome paint probe, and capable to form micronuclei *in vitro* after exposure to colchicine. The transferred chromosome was active, as was demonstrated through the survival C12 and B1 clones in HAT selective medium, because of the presence of an active HPRT gene. We also analyzed the chromosomal distribution for each clone obtained, and as we expected in each clone analyzed the median chromosome distribution was different, according to the random chromosome loss occurred after the fusion.

In the second step of the work, our goal was to transfer the normal X chromosome from the chromosomal donor HPRT+ line (A9/MEF clone) to the recipient HPRT- cell line (HM1). We selected the C12 hybrid clone as donor cell line due to its better ability to form micronuclei *in vitro*, despite having only one X chromosome and thus a lower probability to give rise the transfer than the B1 clone.

We set a microcell-mediated chromosome transfer (MMCT) protocol, adjusting the time of colchicine exposure and the time of cell fusion using the PEG fusogen. Indeed, after 48 h of C12 donor clone incubation with colchicine, we observed the highest number of micronuclei. Subsequently, after cytochalasin B digestion in a Percoll gradient centrifugation and subsequent filtration we obtained a crude microcell suspension completely free of A9 whole cells or large microcells consisting of more than one chromosome. Using small pore filters it was possible to collect the smaller microcells, then assumed carrying a single chromosome, compared to larger ones. Another critical point to be set was the PEG concentration and time of exposure. It is known that the optimal PEG concentration in culture ranges from 35% to 50%, because a lower concentration is better tolerate by cells but is also less efficient. So we set these parameters in an optimal way: 50% PEG for 1 min.

All these conditions were used to perform the MMCT protocol.

After obtaining the optimum microcells suspension, we fused these with an equal amount of monodispersed parental HM1 cells, and we re-plated the cells. The day after, the HAT selective medium was added. We obtained two HAT-resistant clones, HM1 cl8 and cl1, both carrying a HM1-like structure, able to survive in HAT selective medium. By using C12 donor clone, our MMCT procedure was shown to have an efficiency of about $1/10^{-7}$. The efficiency of chromosome transfer was measured as the ratio between the number of HAT resistant clones and the number of microcells used.

The confirmation of the chromosome transfer in the recipient cell line, bearing the functional HPRT gene, was obtained by genomic HPRT PCR. Both MMCT clones obtained demonstrated the presence of both allelic forms of the gene, the mutated and the wild-type alleles. By chromosome distribution analysis we observed that our MMCT fusion protocol permitted the transfer of only one chromosome into the recipient line, as both clones showed a karyotype 2n =41 chromosomes.

Several reports of a single chromosome transfer have been present in literature, but many of them have not been characterized in detail by using cytogenetic and molecular technique. Therefore we performed a more detailed analysis on the HM1 clone 8 (cl8), which has showed greater chromosome stability *in vitro*. Analysis of the chromosome distribution, in fact, was performed both in cl8 and in the cl1 clones, and we found that in the first one the chromosome distribution was more stable, both by adding and removing the HAT selective medium (2n=41).

To confirm the presence of two X chromosomes originating from two different parental lines we performed several genetic analyses.

After showing by PCR the presence of both the mutated and the normal HPRT gene, we analyzed some SNP polymorphisms along the X chromosome to confirm the presence of a supernumerary chromosome derived from a different strain. We selected, in the C57 SNP database of NCBI, SNPs informative for our purpose and we investigated these in the clone. As expected, both the SNPs of cell donor line and the SNPs of the recipient cell line were present. This data, together with the presence of both allelic forms of the HPRT gene confirm the acquisition of an additional MEF-derived X chromosome.

Moreover, both the karyotype analysis and molecular cytogenetic approaches using FISH and M-FISH revealed the absence of rearrangements or aneuploidy in the MMCT cl8 genome. It is important to point out that through the classical DAPI banding the limit of resolution is about 2Mb, so the complete cytogenetic analysis was given by using the M-FISH technique, which ruled out translocations in the genome. Our approach, then, was shown to cure a genetic defect without causing macroscopic chromosome rearrangements in the recipient cell line. We can therefore conclude that our protocol allowed the maintenance of a normal karyotype, because we did not find, in the analyzed metaphases, rearrangements with the exception of the desired additional X chromosome.

Finally, we wanted to show that our microcell fusion protocol does not alter the pluripotency of recipient cells used. Indeed, the analysis we conducted on the MMCT cl8 showed the ability to form teratomas *in vivo*, just like the HM1 parental line. Following subcutaneous inoculum of MMCT cl8 cells in immuno-compromised mice we obtained a well-differentiated teratoma. In fact, hematoxylin-eosin stained histological samples showed derivatives from all the three primordial germ layers, mesoderm, endoderm and ectoderm; this means that following MMCT, clones retained the ability to differentiate *in vitro.*

Upon completion of this assay we also investigated the maintenance of pluripotency through the presence of alkaline phosphatase activity and the presence of classical stem cell markers, the latter both analyzed using cDNA PCR experiment and immunofluorescent assay. The presence of phosphatase alkaline activity is considered the first useful test to demonstrate the pluripotency of a cell line, and as expected the clone showed an activity comparable to the parental line. Similarly, the classic stemness markers investigated were found positive, by both RT-PCR and immunofluorescence analysis. Although the latter is a qualitative test, it can be stated that the presence of the Nanog, Oct4 and SOX-2 markers is apparently comparable to

that found in the parental HM1 cell line. All data collected allowed us to conclude that our protocol MMCT fusion did not affect the pluripotency of the cells used.

6. CONCLUSIONS

Several gene therapy approaches on genetic hereditary disease have been tested, including those based on homologous recombination and viral vectors. Because of the limits regarding these techniques, no strategy can be considered safe and efficient in all the clinical situations.

Our gene therapy approach, by using MMCT, can be considered relatively innovative and safe. The transfer of an exogenous X chromosome into mutated cells has been not shown to cause chromosome rearrangements, involving autosomes or sex chromosomes. In the protocol we used, a whole X chromosome was transferred to a recipient HPRT-defective cell line, and this led to a supernumerary X chromosome-containing cell line. This does not represent a problem, in that aneuploidies involving the number of X chromosomes in literature are well described and are compatible with adult life, since the additional X chromosomes are inactivated in cells for dosage compensation.

For our project we used the Lesch-Nyhan disease as a model of X-linked disease, but according to the data obtained, our MMCT protocol can be considered as an *in vitro* cell model to potentially cure all X-linked diseases. We can therefore say that, by using this protocol, we have demonstrated the capability to cure a genetic defect, leading to the complete rescue of the disease, without inducing chromosome rearrangements or insertional mutagenesis and conserving intact all the regulatory regions needed for a correct gene expression.

Moreover, with regard to applications based on stem cells, it is noteworthy that the procedure did not alter the pluripotency of parental cells. This suggests that iPSC could be modified by MMCT and subsequently induced to differentiate toward the tissues needed for a specific disease. Our long-term goal is to allow the applicability of the method to cell therapy.

We think that also in the field of synthetic biology, the possibility to transfer an intact single chromosome could be interesting. This could be relevant if the results by the Venter group will have to be extended to mammalian

chromosomes, although for this moment this appears to be a goal very far away.

7. REFERENCES

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Acknowledgements

First of all, thanks to Dr.Paolo Vezzoni and Dr.ssa Anna Villa for giving me the opportunity to work in this group, infusing me the pleasure of research.

Thanks to all other people of the laboratory, which more or less helped and taught me over these years; in particular Marianna, Lucia and Elena.. thanks for your teaching and for the technical and moral support.