TITLE: THE MYELOMA STEM CELL CONCEPT - REVISITED

Background: Multiple Myeloma (MM) is a neoplasia defined by the accumulation of malignant plasma cells in the bone marrow. Many recent therapeutic advances have extended survival, but are not curative and new approaches are being sought that can underpin disease resolution. Understanding pathogenesis in MM will benefit these searches. Although malignant cells, which represent terminally differentiated B-cells, are readily identifiable by morphological criteria, it is as yet not known with certainty whether a less differentiated cell may actually be feeding tumor growth. This uncertainty has arisen due to a number of observations suggestive of a less mature clonal precursor. More recently the question of whether such a 'feeder' cell may in fact represent one or more clonal 'stem' cell had also emerged based on 'the stepwise oncogenesis' and as in other cancers, could represent a paradigm shift in understanding MM origins and progression. The recent focus on potential stem cells in cancer has generated much interest and rapid translational investigations including novel biotechnologies.

The Cancer Stem Cell Concept: One issue that has emerged in this new field is the precise definition of a stem cell in malignancy. As a general description, cancer stem cells (CSCs) have been proposed as a relatively minor subpopulation of low frequent cells that are less differentiated than the bulk of tumor cells within a heterogeneous clonal hierarchy. CSCs maintain tumor bulk via asynchronous division to yield two pools, one able to self-renew to maintain the CSC pool and the other unable to proliferate or seed tumor growth but adding bulk to the malignant population. The important aspect of CSCs is that such cells have 'stem cell-like properties, especially the capacity for self-renewal'. Other properties of CSCs now described include distinctive signalling pathways and patterns of gene expression and activity reminiscent of normal embryonal or somatic stem cells. Furthermore, a fundamental requirement is to relate the CSC to the cell of origin that gives rise to a given tumor, that is, a cell located in normal lineage development which transforms at a particular stage of development to initiate a tumor. It becomes relevant then to define how such a cell of origin becomes malignant and acquires the mantle of a CSC, invoking genetic or epigenetic lesions as well as cellular changes. Clearly, defining CSCs presents challenges. In relation to MM, these challenges become magnified.

Objectives: Cells belonging to the Myeloma clone (termed clonotypic cells) that precede the PC stage have been identified in peripheral blood and BM. The earliest clonotypic cells identified shows a preswitch isotype and were exclusively found in the CD19+/CD38- B cell compartment suggesting that the clonotypic precursors is a memory B-cell. Whether clonal precursor cells are fully transformed with a clonogenic potential remains a controversial issue with studies supporting the idea of a MM "stem-cell" present in a preplasmacytic compartment, contrasted by studies unable to identify a clonogenic potential. Disease characterization has revealed a number of phenotypic and molecular features
including recurrent mutations that support the existence of a clonally-related 'less mature' cell, and the question arises whether this may include Myeloma Stem Cells (MSC) – which are critical to and harbour specific molecular genetic events propagating the malignancy. To progress work in this area, Myeloma researchers from the European Myeloma Network established the Myeloma stem cell network (MSCNET), which has set out to identify the nature of the cell underlying disease origin and persistence.

Hypothesis and aims: Our *hypothesis* is that the MSC can be defined and characterized to underpin effective targeted therapy in MM. In this disease, the current state of the art suggests that a preplasmablastic CD19+CD20+CD138- cell (B-MSC) may exist as a putative stem cell. However, there is evidence which points to an alternative view, that the MSC may be a CD19-CD20-CD138+ plasmablast/plasma cell (P-MSC). It is conceivable that both or even more stem cell components may be relevant for the oncogene hits responsible for MM progression. This MSCNET translational programme has extended our current efforts to characterize the potential stem cell compartments in MM. To this end, our *specific aims* have been to

1) Develop protocols to identify and isolate the potential MSC compartments;

2) Investigate the function of such cells to characterize these as CSCs being responsible for disease persistence and evolution;
3) Define MSC-associated characteristics for future targeted therapy.
Material and Methods: Following a range of scientific meetings and technical workshops we now share the relevant competences and experiences related to multiparametric flow cytometry (MFC), real time PCR assays, cDNA-Chip and PepChip technology and storage of biological samples with specific clinical information. Furthermore we share models for in vitro differentiation of B cells and an animal model for in vivo transplantation studies. By these platforms we have generated a common database with analytic information about potential B lineage subsets (naïve, large centroblast, small centroblast, centrocytes, memory B-cell and plasmablast/cells) in peripheral blood and bone marrow of impact for pathogenesis, including a well characterized panel of Myeloma cancer cell lines. Potential stem cell genes have been identified in the current literature and available databases and PCR assays established for single cell (N=1-100) analysis for stem cell genes, transcription factors and differentiation markers.
Major results: First, in a model of light-chain MM (LC-MM), where CD138+ tumor cells express only the light chain, we sought molecular evidence for less mature cells bearing both Ig heavy and light chains, in different B-cell subsets (naïve, IgM+, IgM- B-cells, plasmablasts, plasma cells). In 5/6 cases, no evidence was found for such a cell, and in the remaining cases, IgH and L chain expression was restricted to the CD138+ fraction, suggesting that the progenitor cell in LCMM is a plasmablast/plasma cell.
Second, in a model of Ras mutated MM patients studied by single cell cDNA libraries, we identified that memory B-cells do lacks ”late" oncogene like (K-RAS) mutations but express the ”early" oncogene presumably representing a Clonotypic remnant that is only partially transformed. This demonstrated that CD19+/38- cells do not but CD19-/38+ cells harbour the Ras mutated clone. This observation and the indications from the study of LC MM indicate a stepwise evolution of MSC within the Myeloma hierarchy in parallel with the step wise oncogenesis.
Third, in the 5T2 and 5T33 mice model we have succeeded in identification of tumour V(D)J transcripts ready for transplantation experiments. In the mouse transplantation model CD138+ cells rapidly engrafted disease (3-4 weeks), whereas CD138- cell mediated engraftment was delayed (4-12 weeks), which was supported by in vitro clonogenic assay. This demonstrated that the marker CD138 is not sufficient to identify a Myeloma stem cell.
Fourth, in a study of 18 Myeloma cell lines we determined the frequency of culture initiating cells concluding that CD138+ cells had a 10-100 fold higher frequency than CD138- cells. This demonstrated that the marker CD138 is not sufficient to identify Myeloma cell line culture initiating cells.
Conclusions: MSCNET sought to initiate detailed studies to define both the B-MSC and PMSC, in primary tumour samples as well as in disease models. With regards to the B-MSC, we were unable to identify clonally-derived progeny in distinct B-cell subsets representative of several maturation stages leading to plasma cells, and this study has been accepted as a Speaker Presentation at ASH 2010, the foremost platform for cutting-edge research in Haematology. We identified a clonal hierarchy in 5T murine Myeloma, and showed that CD138+ malignant plasma cells are sufficient to propagate tumor growth. In this model however, the ‘stem cell’ component appears more complex, as CD138- cells can also engraft, although at a slower rate and this question will be addressed in future collaborations among MSCNET members, directly facilitated by the network’s activities during tenure of this program. In relation to the oncogenic hits driving ‘stem cells’, MSCNET has provided pertinent state-of-art observations. Our study on LC-MM raises the conceptual issue of a sIg+ B-MSC as being an unlikely feeder cell in this form of disease, as the genetic lesions that such a cell would need to acquire to feed the light chain phenotype on a cyclic basis (as proposed by the CSC paradigm) appears highly unlikely. The analysis of KRAS mutations in memory B-cells that are aberrant, and therefore tumor-derived, has provided pivotal insights in agreement with our LC-MM predictions. This data showed that KRAS mutations are only found in malignant plasma cells, not memory Bcells (the B-MSC counterpart). This cements

the proposal that a MSC which is a B-MSC is highly unlikely to acquire the repertoire of mutations to establish features associated with presentation disease every time it cycles to feed the tumor bulk. As argued in the KRAS study, this B-MSC appear to be a residue of the cell of origin giving rise to MM, but appear to have no malignant potential.
In conclusion, MSCNET favours the P-MSC model as driving malignant growth and has delineated multiple genetic pathways by which malignant PCs can support this potential, which currently is clinical validated.