What's up NF1?

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Embryonic stem (ES) cells are multipotent. ES cell lines can be established from wild-type and mutant pre-implantation mammal embryos and genetically manipulated in vitro and then used to mimic disease-associated phenotypes in order to decipher the cellular (including proliferation, specification and differentiation) and molecular mechanisms affected by mutations. These cells may be pushed to undergo differentiation using different procedures including (i) injecting them into blastocoel to allow development in utero, (ii) grafting them into mice for teratoma formation, (iii) inserting them into chicken embryos for further development, and (iv) in vitro. Using these different methods of differentiation has allowed production of various tissues and cell types, including melanocytes. This required the use of various combinations of factors, including extracellular matrix (such as fibronectin), growth factors (such as BMP4, WNT3A, SCF and EDN3), antioxidants (such as ascorbic acid) and feeder layers (Fang et al., 2006; Nissan et al., 2011; Pla et al., 2004). Under these conditions, pluripotency markers, such as OCT4, NANOG and SOX2, decrease, whereas pigment cell markers, such as TYR, TYRP1 and MITF, increase, resulting in pigmented cells. These ES-derived melanocytes can then be evaluated for their ability to produce melanin and to transfer their melanosomes to cocultured keratinocytes (Nissan et al., 2011).

Neurofibromatosis type 1 is a monogenic autosomal dominant disorder of the group of RASopathies, caused by alterations in the neurofibromin gene *NF1*. NF1 is a GTPase-activating protein (GAP) that negatively regulates the MAPK pathway and is considered to be a tumour suppressor. The symptoms of neurofibromatosis type 1 include non-malignant clinical features, such as abnormal pigmentation (café-au-lait macules [CALMs] and axillary freckling), skeletal and cardiovascular deformities, but also tumours, such as neurofibroma, glioma, peripheral nerve sheath tumours, breast cancer, phaeochromocytoma and lymphoma. The importance of NF1 during embryonic development is emerging from mouse molecular genetics. Mice heterozygous for NF1 have no relevant phenotype (including coat colour), but mice lacking NF1 die during embryonic development, reflecting the critical role of neurofibromin in tissue development and organogenesis. In humans, it is difficult to evaluate the importance of NF1 during embryonic development in vivo, for obvious reasons.

Here, Allouche and colleagues established two human ES cell lines derived from embryos carrying a heterozygous mutation of the NF1 gene, a deletion of four nucleotides leading to the creation of an early stop codon. The wild-type allele produces a 2818-amino acid protein, whereas the mutated allele encodes a 1156-amino acid protein. The mutated version lacks almost two-thirds of the wild-type NF1 protein, including the GRD (GAP-related domain) and all the subsequent structures important for protein–protein interactions. The differentiation of two NF1 and two unrelated wild-type ES cell lines into melanocytes was induced, generating four corresponding melanocyte cell lines. The melanocytes of all genotypes looked alike, morphologically and molecularly, and they all expressed appropriate pigment cell-specific markers. These observations fit nicely with the information that we have on the histology of café-au-lait macules and associated melanocytes, as no morphological modifications of the cells was revealed. However, an increment in the number of melanocytes and a higher production of melanin were observed.

The absence of functional NF1 did not affect the overall differentiation of ES cells into melanocytes. Once mel-NF and mel-WT cells had been established, a lack of functional NF1 was found to be associated with higher rates of proliferation in the differentiated cells and hyperpigmentation.

The NF1 protein levels of mel-NF cells were found to be about half of the levels of mel-WT cells. The authors then quantified the proteins involved in melanogenesis, the amount of melanin and the type of melanosomes present in these melanocytes. They found that mel-NF cells contained higher levels of MITF, TYR and DCT than mel-WT cells, by factors of two, three and five, respectively. Similarly, lower levels of NF1 were associated with higher levels of melanin. Mel-NF cells contained 60% stage III and IV melanosomes (late stage melanosome development), a much higher proportion than the 10% in mel-WT cells. NF1 knockdown in mel-WT cells yielded mel-WT-siNF1 cells with similar characteristics to mel-NF1 cells. These results indicate that, but not in the establishment of melanocytes after in vitro differentiation from ES cells. As the differentiation of melanocytes was unaffected, similar results would be expected for melanocytes established from non-CALM NF1 (showing no pigmentation, but a priori heterozygous for NF1) and WT patients, although such results are not yet available.

NF1 is involved in proliferation, melanin production and the maturation of melanosomes in melanocytes

However, melanocytes from CALMs have been shown to harbour a second hit in the *NF1* gene (De Schepper et al., 2008). Allouche and co-workers mimicked CALMs in established mel-NF1 cells, by decreasing the amount of NF1 in mel-NF1 cells (80% knockdown). This decrease in NF1 levels led to an expected increase in melanin content and tyrosinase levels. These findings raise questions about the consequences for melanocyte differentiation of knocking down or knocking out NF1 expression in NF1-ES cells. In other words, are CALMs due to homozygous NF1 microchimerism in these patients or simply to a second hit in NF1?

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Given the importance of NF1 in the MAPK and cAMP pathways, Allouche et al. performed a series of experiments to explore changes in these pathways due to reduced NF1 protein. They found that phospho-ERK levels in mel-NF1 cells were twice those in mel-WT cells and that cAMP levels in mel-NF1 cells were four times those in mel-WT cells. Similar results were obtained when NF1 expression was knocked down in mel-NF1 cells. Next, the authors performed a rescue experiment to investigate whether the defective melanogenesis observed in NF1 human ES-derived melanocytes could be restored/repaired. A pharmacological approach was also used, with inhibitors of MEK (PD032059), PKA-cAMP (HA1004) and tyrosinase (kojic acid), leading to a reduction of the associated pathways and melanin as well as downregulation of key enzymes involved in melanogenesis. From these results, Allouche and colleagues concluded that the effect on melanogenesis of decreased NF1 expression is probably due to the ERK and cAMP signalling pathways and that it is possible to block these signalling pathways pharmacologically and reduce hyperpigmentation.

In a nutshell, a decrease in NF1 levels resulted in an upregulation of the cAMP and MAPK pathways, leading to hyperpigmentation explained by an upregulation of markers of the melanocyte lineage and an increase in melanin content. This study demonstrates the importance of human ES cells for mimicking human diseases and provides support for the therapeutic potential of these cells as a cellular model. Even though hyperpigmentation observed in CALMs is not life-threatening, it does impact the quality of life of patients, so that it might be worth exploring the effects of topical treatment with specific tyrosinase inhibitors.

Much attention has recently focused on NF1, not only because of neurofibromatosis type 1, but also because of the involvement of this protein in melanoma. NF1 has recently been highlighted as characteristic of one out of four distinct classes of human melanoma, along with BRAF, NRAS and 'triple-WT' subtypes ('Genomic Classification of Cutaneous Melanoma', 2015). Endless opportunities are available using ES cells to mimic human diseases. Allouche and colleagues chose NF1 and demonstrated that the reduction of NF1 leads to upregulation of key signalling pathways affecting melanogenesis. However, it is also important to investigate the role of NF1 in melanomagenesis, and therefore, as always, further studies are needed to decipher the role of NF1. Clearly, with this publication, the authors made a step in the right direction laying the groundwork to continue on it.

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Perturbing resistance: a network perspective

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The recent convergence of high-dimensional molecular datasets with small-molecule inhibitor pipelines allows for selective targeting of aberrantly regulated pathways in many cancer types. But intratumor heterogeneity, paradoxical activation, intrinsic or acquired therapeutic resistance, and nonlinear pathway interactions confound most simple targeting strategies (Widmer et al., 2015).

For instance, MAPK signaling is activated by hot spot mutations in BRAF, which are found in about 40–50% of melanoma cases. Although the BRAF inhibitors vemurafenib and dabrafenib have been incredibly effective in the clinic, sustained long-term responses to single-agent therapy have been rare. The combination of BRAF and MEK inhibitors, such as trametinib and cobimetinib, has prolonged response duration and improved overall survival, but most patients still progress after ~1 year of therapy (Robert et al., 2015). Thus, the mechanisms of therapeutic resistance are under intense investigation at the moment, with molecularly driven rational combinations of more than two targeted therapies already in clinical trials. The challenge, however, is in using extensive molecular data to identify who might benefit from which drug combinations or treatment schedules.

The Systems Biology paradigm offers a solution to this problem by generating testable models of the dynamic network properties of cells that improve on a purely reductionist approach by accounting for higher level interactions between system components (Levesque and Benfey, 2004). An excellent example was recently published in the open-access journal eLife. The authors followed previous work in which they

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