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Biochips for Regenerative Medicine: Real-time Stem Cell Continuous Monitoring as Inferred by High-Throughput Gene Analysis

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Abstract Regenerative medicine is a novel clinical branch aiming at the cure of diseases by replacement of damaged tissues. The crucial use of stem cells makes this area rich of challenges, given the poorly understood mechanisms of differentiation. One highly needed and yet unavailable technology should allow us to monitor the exact (metabolic) state of stem cells differentiation to maximize the effectiveness of their implant in vivo. This is challenged by the fact that not all relevant metabolites in stem cells differentiation are known and not all metabolites can currently be continuously monitored. To bring advancements in this direction, we propose the enhancement and integration of two available technologies into a general pipeline. Namely, high-throughput biochip for gene expression screening to pre-select the variables that are most likely to be relevant in the identification of the stem cells' state and low-throughput biochip for continuous monitoring of cell metabolism with highly sensitive carbon nanotubes-based sensors. Intriguingly, additionally to the involvement of multidisciplinary expertise (medicine, molecular biology, computer science, engineering, and physics), this whole query heavily relies on biochips: it starts in fact from the use of high-throughput ones, which output, in turn, becomes the base for the design of low-throughput, highly sensitive biochips. Future research is warranted in this direction to develop and validated the proposed device.

Keywords High-throughput biology · Nano-structured biochip · Stem cell differentiation · Metabolic pathways/markers

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1 Introduction

Regenerative medicine is among the most promising and challenging research areas related to health. Succinctly speaking, it involves the introduction in vivo of newly/partially differentiated cells into damaged tissues, that can therefore regenerate and repair whole

organs, or in vitro generation of tissues and organs. Pioneering work applied to cardiac surgery on ischemic areas has generated great hopes as well as novel issues [1]. Due to the crucial use of stem cells, challenges range from the difficulties arising from ethical concerns regarding embryonic stem cells—that limit the possible systems under study—to the complexity of the process of differentiation and the number of variables involved in cells' fate. Therefore, it is of the utmost importance to offer technologies instrumental to the deciphering of such complexity. To meet this goal, a variety of technologies and of backgrounds are necessary to cope with the emerging difficulties. In particular, identifying the main processes involved in the differentiation and monitoring in real time, the evolution of such processes can constitute a crucial advancement in this area since one pre-requisite for cells implant is the proper and precise knowledge of their state. Intriguingly, the quest for such information can take the spirally formed shape of a path where biochips play a crucial role. This path originates in the development of high-throughput biochips allowing the identification of molecules of interest that can serve as markers for biological processes' further monitoring (see Fig. 1). Before discussing the pipeline proposed in this article to perform this integration, we briefly recollect the state of the art on the technologies we will base our approach on.

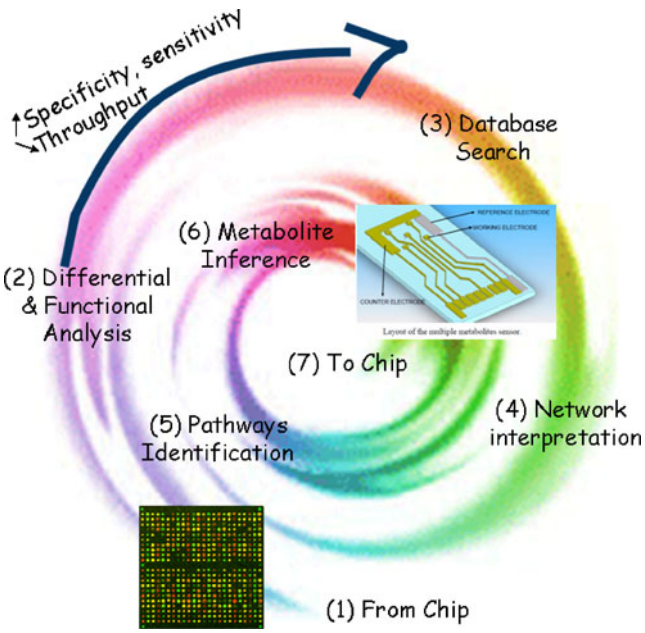

Fig. 1 Depicts an ideal path of biochips evolution, showing increasing precision and decreasing throughput. The various steps are described along this article. In particular, the first three steps, representing standard elaboration of high-throughput biochip, are described in Section 2, and the last three that are typical of this approach as pre-processing for nanobiochip design, in Section 3

Microarrays for gene expression are the molecular biochips (appeared roughly 15–20 years ago [2]) that have revolutionized biology, by leading to the generation of novel scientific branches like bioinformatics and computational biology. Although soon-to-be overshaded by sequencing technologies [3, 4], their use in differential analyses (i.e., identification of genome-wide variations in expression among samples taken from different patients, after different treatments, at different time points, etc.) remains a yet cheaper and valuable approach [5, 6]. The technical solutions used to perform parallel hybridization are numerous but can be grouped in two main categories, notably for the choice of the blueprint molecules on the chip. In the first type, they are fragments of cellular extracts and their length goes from 20 to hundreds of base pairs (named *two channels* since they perform the comparative analysis of the molecules abundance in two cells populations); in the second type, they are synthesized short probes (15–25 bp) of known sequence which are able to capture specific fragments (*single channel*). The fragments diluted in the sample solution are pre-processed and coupled with a permanent marker molecule which, once the fragment has been captured by a complementary strand on the surface of the chip, is able to generate a localized detectable signal (hence, the well-known microarray heatmaps). Although the reproducibility of the results is not perfect, the MicroArray Quality Control Project, led by the US Food and Drug Administration [7] proved that findings can be shown to overlap, provided great care is used in the definition of the experimental protocols, and that results are compared in terms of genes functionalities.

Conversely to the high-throughput snapshot offered by microarrays, biosensors technology provides the direct and continuous monitoring of a selected number of metabolites. With respect to the variables to be sensed, biosensors based on oxidases have already been successfully proposed for the continuous monitoring of glucose [8, 9] and lactate [10], two highly relevant molecules in cell metabolism, that have also been used to monitor cell cultures during growth and differentiation [11]. Recent advancements have enabled the remote monitoring of such molecules in both humans [12] and animal models [13]. Additionally, biochips-sensing metabolites related to P450-based metabolic pathways have been demonstrated to be reliable [14] even in the case of multiple substrates interacting with the same cytochromes [15].

The combination of electrochemical detection for metabolites sensing with carbon nanotubes has proven

to remarkably enhance detection [16] and this approach has recently been extended to sensing other metabolites, e.g., ATP [17].

1.1 The Proposed Biochip Pipeline

In this work, the distinctive characteristic of microarrays, i.e., the bird's eye, systemic view they offer, is used as a pre-condition to identify the main molecular variables interested in stem cells differentiation. As depicted intuitively in Fig. 1, the derived information is processed with bioinformatics techniques (differential and network analysis [18, 19]) to identify the genes that show statistically significant variations in their expression when comparing different states of the cell. This information (i.e., a set of differentially expressed genes), is then elaborated via integration with a variety of biological databases, to generate a network of interactions from the list of genes. Finally, the manual curation allows to identify the pathways that are more relevant, and from there to infer the metabolites produced, and therefore the likely markers to be monitored: these metabolites, in fact, can ultimately serve as sensing variable in a low-throughput carbon nanotube-based biochip.

The whole chain of events (molecules identification, selection, and sensing) remains in the realm of molecular biology, where, high-throughput biology serves as a base for the generation of advanced and focused tools for the online monitoring of stem cells differentiation. In our work, the conditions tested are embodied by two states of rats pluripotent embryonic stem cells (ESCs). ESCs have the capability to unlimitedly proliferate in vitro maintaining an undifferentiated state. Due to their pluripotentiality, they retain the ability to differentiate into all cell types in vivo and can be induced to form derivatives of all three germ layers (ectoderm, endoderm and mesoderm) in vitro. The pluripotential properties of such cells can be assessed by the expression of ESC markers such as placental alkalin phpsphatase (AP), SSEA-1, SSEA-3, SSEA-4, POU transcription factor octamer-4 (Oct-4), TRA-1-60, and TRA-1-81 (1,2). When cultured in the absence of feeder layer in no leukaemia inhibiting factor (LIF) containing medium, rats ESC (RESCs) are induced to form large and spherical cells aggregates known as embryoid bodies (EBs) [20]. After some days in culture, these EBs can be processed by enzymatic and/or mechanical dissociation to obtain a single cell suspension. If plated on appropriate coated substrates, cells attach and upon removal of growth factors differentiate; when ESCs start to differentiate, the indicated pluripotency markers disappear. Using this culture system, we can obtain both Oct-4 high-expressing cells The cells we processed were both single cells, thus true ESCs, and Oct-4 low-expressing cells. Therefore, we can speculate to have a very early differentiation stage from our Oct-4-positive cells.

The elucidation of the stem cells metabolism during differentiation and especially at the early phases of such a transition could lead to a better understanding of embryonic stem cells behavior and to the identification of crucial markers for monitoring their behavior. Our aim was to mark a transcriptional difference between two close developmental states distinguishable for the expression of Oct-4. It is generally accepted that Oct-4 is one of the 3 crucial molecules in the core regulatory network maintaining the ESC pluripotency [21]. Thus, this cell system is a preferential candidate to study possible metabolic markers (and biosensors targeting molecules of metabolic interest) able to monitor staminality and/or differentiation.

On the sensor side, the pathways mapped by the differential and networking analysis can be screened (using number of databases [18, 22]) in search of enzymes that play a significant role in metabolic regulation. For example, some oxidases are involved, with a number of common molecules (such as glucose, lactate, glutamate, etc.), in reactions that have a key role in cell metabolism. Similarly, enzymes of the superfamily of the cytochromes P450 are involved in the metabolism of both endogenous and exogenous metabolism in all eukaryotic organism [14]. Such classes of enzymes link the genes identified to be relevant in the differentiation process, with the molecules that can be sensed to monitor such differentiation, and therefore they are crucial toward the development of low-throughput biochip for continuous cell monitoring.

2 Materials and Methods

2.1 Cell Culture and mRNA Collection

RESCs were cultured as clusters for two passages on mouse embryonic fibroblasts (MEF) and one passage without MEF from thawing. Afterwards, single cell suspensions were derived and cultured for further two passages in SCML medium. SCML medium was prepared as described by Turksen [23] and it was supplemented with 4 ng/ml bFGF and 2×103 U/ml LIF. At this stage, Oct-4 high-expressing cells were collected or further cultured for 10 days without bFGF and LIF to yield Oct-4 low-expressing cells. Embryonic stem cells were cultured under two conditions: the Oct-4-positive and Oct-4-negative state. For each condition,

three replicates where obtained in two different batches of experiments (these were processed in three different cell batches; two were screened together and one subsequently, with microarray for gene expression). RNA were extracted from single cells cultured with or without mitogens with miRNAeasy mini kit (Qiagen, Milan, Italy) according to the recommendation of the manufacturer.

2.2 Microarrays for Gene Expression

Microarrays experiments (one channel technology) were performed at Genopode (http://www.unil.ch/acces/page36639_fr.html), using Affymetrix Rat Gene 1.0 ST arrays, with 27,342 transcripts, according to the manufacturer's instructions.

2.3 Differential Analysis

The raw microarray data were pre-processed using affymetrix power tools with RMA normalization. The three replicates were obtained from two batches, therefore the analysis was performed within and between batches to assess the entity of the bias due to the batch effect. For the two microarray samples from experiment of round 1, fold change was used to select differentially expressed probes, cut-off set to two [24]. For the four microarray samples from experiment of round 2 and the combined six microarray samples (all data analysis) since there were only few replicates (2–5/group), the moderated *t*-statistics method [25], making use of the *limma* R-package was adopted. Probes with p values of ≤ 0.001 were selected to be statistically significant. As an additional filtering criteria, we removed all the differentially expressed probes with average expression <4.5, as well as the ones showing no mRNA annotation.

3 Results and Discussion

The results of the differential analysis lead to the identification of the genes that have undergone important changes during the experiment, i.e., before and after early differentiation (Oct-4 high-/low-expressing state).

The results in this form consist of an annotated list of meaningful genes. However, in order to achieve information relevant for the identification of the metabolites that may be involved in this early step of the differentiation, another type of output, able to capture the complexity of the genes interactions, is necessary. In particular, the representation of genes interconnections, using a graph, appears to be suitable for this problem and is commonly used in computational biology. A graph is a set $G = \{N, E\}$, where G represents the set of nodes (i.e., annotated genes) and E the set of edges (that is the connections occurring among these genes). Computational biology is concerned with multiple way of reconstructing such graphs, and the most appropriate for the current experimental design relies on literature databases where number of connections are listed, based on a variety of published experiments. In particular, STRING [18] offers the needed flexibility to draw this network. STRING is a database of known and predicted protein interactions including direct (physical) and indirect (functional) associations derived from four sources: genomic context, high-throughput experiments, coexpression and previous knowledge (literature). The four sources can be further extended into eight types of evidences for associations while in the resulting map, different line colors represent the types of evidence. The confidence score is an indicator of the robustness of the connection and is set to medium by default.

In particular, Oct-4 downregulation following mitogens withdrawal results in the differential expression of $\tilde{1}20$ relevant genes (listed in Supplementary Information) which resulting network, processed with STRING, is shown in Fig. 2. Two very interesting clusters of genes around insulin-like growth factor (IGF)-1 and TGF-b and related receptors are identified. IGF-1 which we found to be downregulated after Oct-4 downregulation and reduction of pluripotency is a hormone similar in molecular structure to insulin. In humans, it plays an important role in childhood growth and stimulates anabolic processes also in adults. Both the insulin and IGF-1 receptors (IGF-1R) are expressed in the trophectoderm cells and trophoblast tissue [26]. IGF plays a critical role in promotion of survival and proliferation in a diversity of cell types, including both embryonic and adult stem cells. It was also recently shown that self renewal of human embryonic stem cells requires insulin-like IGF-1R [27] and that IGF could act as a co-stimulatory factor for reprogramming somatic cells into induced pluripotent stem cells [28]. These evidences match with our observation that IGF-1 downregulation occurs during Oct-4 downregulation and consequent decline of RESC pluripotency. On the basis of the crucial and complex role of IGF-1 in the regulation of cell differentiation, survival and proliferation [29] we can speculate that our cellular system undergoes profound modifications related to pluripotency and differentiation when Oct4 is downregulated and thus represents an appropriate model to identify potential molecular targets for stem cell monitoring. Since the metabolic responses of differentiating stem

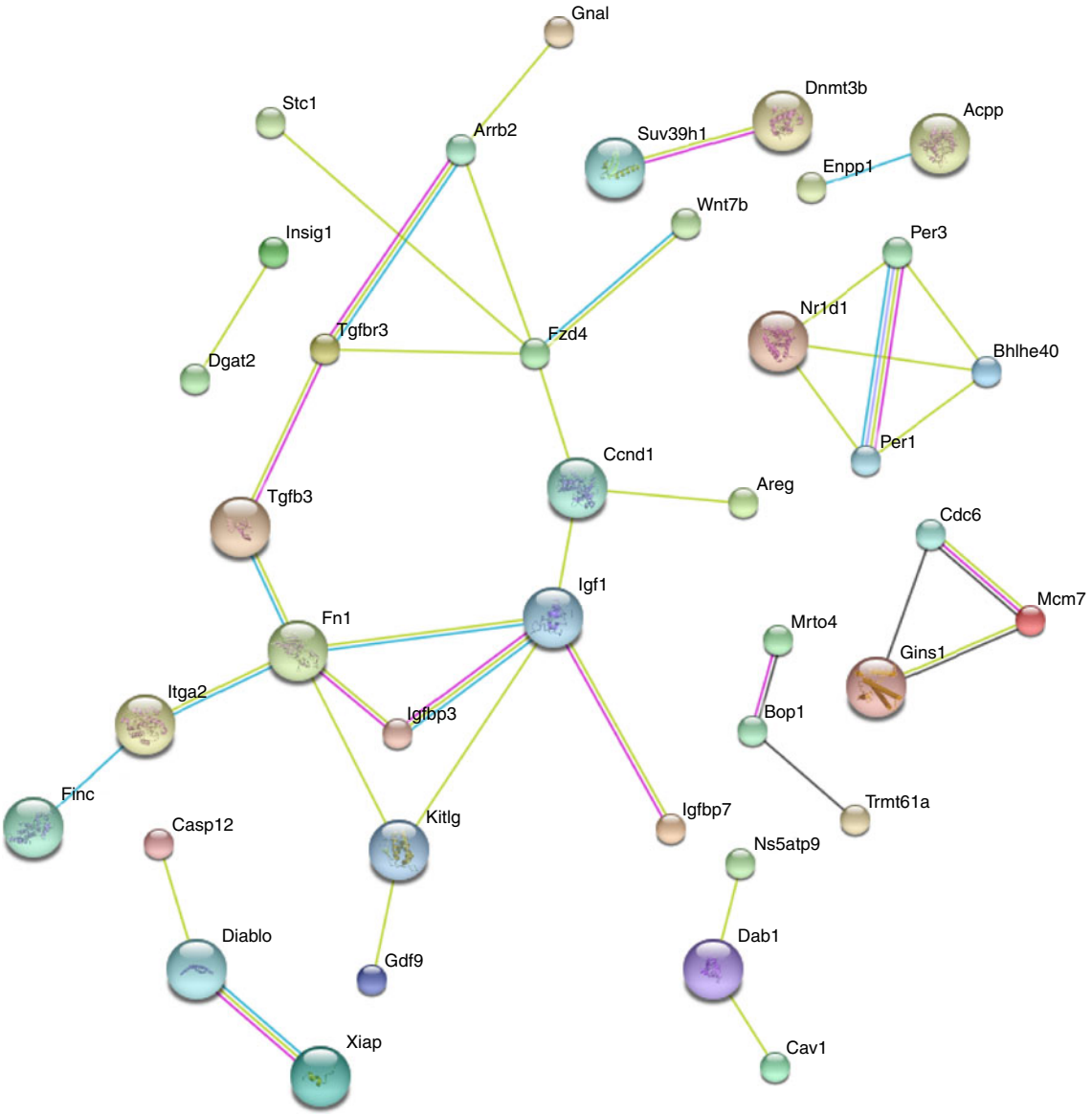

Fig. 2 STRING Network. Color code for edges interpretation: neighborhood (*green*), gene fusion (*red*), cooccurrence (*blue*), coexpression (*dark*), experiments (*pink*), databases (*sky blue*), textming (*kelly*), and homology (*purple*). Only connected genes are depicted, therefore the differentially expressed genes that appeared as isolated nodes where not added in this figure

cells have been poorly investigated despite their potential relevance for the stem cell behavior [30, 31], we focused on the differentially expressed genes which are ontologically related to cellular metabolism and/or encode for enzymes and transporters for extracellular metabolic substrates. In this context, on the basis of its in vivo role in metabolism regulation, the unexplored possibility that IGF-1 differential expression could results in changes in the metabolic state of embryonic stem cells should be investigated, the full list can be found in Online Resource 1. Furthermore, we retrieved an upregulation of Stc1, Per1, Per3, and Insig1 after mitogen withdrawal, for which a clear role in the regulation of the cell metabolism was indicated [32–35]. More generally, we identified subsets of genes in the extracellular region or space whose regulation in the two experimental conditions that we investigated could result in changes of detectable extracellular molecules (i.e., enzymes, and transporters for metabolites, proteins, polysaccharides as well as key detectable molecules such as hormones). In this context, we found a significant upregulation of Adm(adrenomedullin), Acpp (acid phosphatase, prostate), hyaluronoglucosaminidase 1 (Hyal-1),

transcobalamin II (Tcn2), stanniocalcin 1 (Stc1), prostaglandin I2 (prostacyclin) synthase (Ptgis), aldehyde dehydrogenase 6 family member 9 (Aldh6a1), and mannosyl (alpha-1,3-)-glycoprotein beta-1,4-*N*-acetylglucosaminyltransferase (Mgat4a), see Table 1.

This analysis permits the identification of portions of pathways involved in the differentiation and also to highlight in particular genes that appear to be more relevant to our quest. However, an additional step is needed to connect these pathways to the identification of the metabolites that are secreted by the cell and can be sensed as a marker of the cell's state.

Toward this aim, given the clearer signal shown by the variation in expression of the genes in Table 1, we investigated deeper their relation to potential sensing metabolites, in particular searching for connections in the pathways related to the cytochrome P450 superfamily, which will serve as the base of the nanocarbon biosensor. In rat, the enzymes belonging to the P450 superfamily catalyze several metabolic reactions, among which steroid hormone biosynthesis, drug metabolism and linoleic acid metabolism. Since the eight genes identified above are also enzymes, involved in several metabolic reactions, we used the KEGG pathway [22] database to build the connections among them. First, we searched the relationships among the eight genes, by identifying the individual metabolic pathways each gene is involved in. As it is shown in Fig. 3 Acpp is involved in Riboflavin metabolism, Hyal1 in glycosaminoglycan degradation, Aldh6a1 in several pathways such as valine, leucine and isoleucine degradation, Inositol phosphate metabolism and Propanoate metabolism, Mgat4a in *N*-glycan biosynthesis, Ptgis in Arachidonic acid metabolism and Tcn2 in Vitamin digestion and absorption. The last two genes, Adm and Stc1, do not participate in any metabolic pathways.

Table 1 Genes symbols, fold change (logarithm), and gene description of the eight genes found to be significantly upregulated in the early differentiation

Gene symbol	logFC	Gene description
Acpp	1.54	Acid phosphatase, prostate
Hyal-1	1.46	Hyaluronoglucosaminidase 1
Aldh6a1	1.36	Aldehyde dehydrogenase 6 family, member A1
Mgat4a	1.06	Mannosyl (α-1,3-)-glycoprotein β-1,4-*N*-acetylglucosaminyltransferase, isozyme A
Ptgis	1.78	Prostaglandin I2 (prostacyclin) synthase
Tcn2	1.41	Transcobalamin 2
Adm	2.23	Adrenomedullin
Stc1	2.22	Stanniocalcin 1

We then used the whole metabolic pathways of rat to find the connections of the six genes involved in different metabolic pathways, four out of six genes can be linked through serial metabolic reactions. Finally, we added the P450 enzymes—due to their possible use in sensors base—to this map through the shared metabolites. They appear to be related to the highlighted molecules via the caffeine, arachidonic acid, and linoleic acid metabolisms. For examples, Cyp1a2/Cyp2a1/Cyp2a3/Cyp2a2 can all catalyze 1,7-dimethyluric acid to 1,7-dimethylxanthine, and these enzymes can be linked to the original map through a serial metabolic pathways according to the shared metabolite 5-phospho-alpha-D-ribose-1-diphoshphate, a majority of P450 enzymes such as Cyp2c22/Cyp2c13/Cyp2c6 catalyze the metabolic reaction from Linoleate to 12(13)-EpOME and other reactions. Since these proteins all belong to the p450 family, they may share some similar functions, and therefore can catalyze the same reactions. In particular, these enzymes can be connected to the map by the common metabolite D-Glyceraldehyde 3-phosphate and beta-D-fructose 1,6-bisphosphate. Additionally, by direct inspection of Fig. 3, we can identify very interesting connections between the identified genes and molecules such as Lactose and Galactose, that can be easily sensed with assessed nanotechnology. With respect to molecules that act as substrates for the P450 proteins family [36] (see Table 2 for a partial list), we can observe that the ones identified in this study (highlighted in bold) have already been sensed with this approach, and namely: caffeine [37], arachidonic acid [38], arachidonate [39], and linoleic acid [40].

Therefore, this research has highlighted a relevant set of metabolites that can be used to develop a multipanel biochip for monitoring cultured stem cells by following glucose, lactate, caffeine, arachidonic acid, arachidonate, and linoleic acid time trends as molecular markers of the cell metabolism. One actual direction emerging from this analysis is therefore to develop a biochip based on glucose and lactate oxidases as well as on the P450 isoforms shown in Table 2.

Additionally, urate oxidase (Uox) through the purine metabolism pathway and tyrosinase through the riboflavin metabolism pathway (highlighted in purple in the map, Fig. 3) can also bring relevant information. In fact, Uox catalyzes the transformation of uric acid in 5-hydroxyisourate, while purine is synthesized by catalytic reactions that involve several different metabolites, including 5-phospho-D-ribosyl 1-pyrophosphate. The last reacts with glutamine to form 5′-phosphoribosylamine, glutamine, and pyrophosphate. Therefore, uric acid and glutamine are

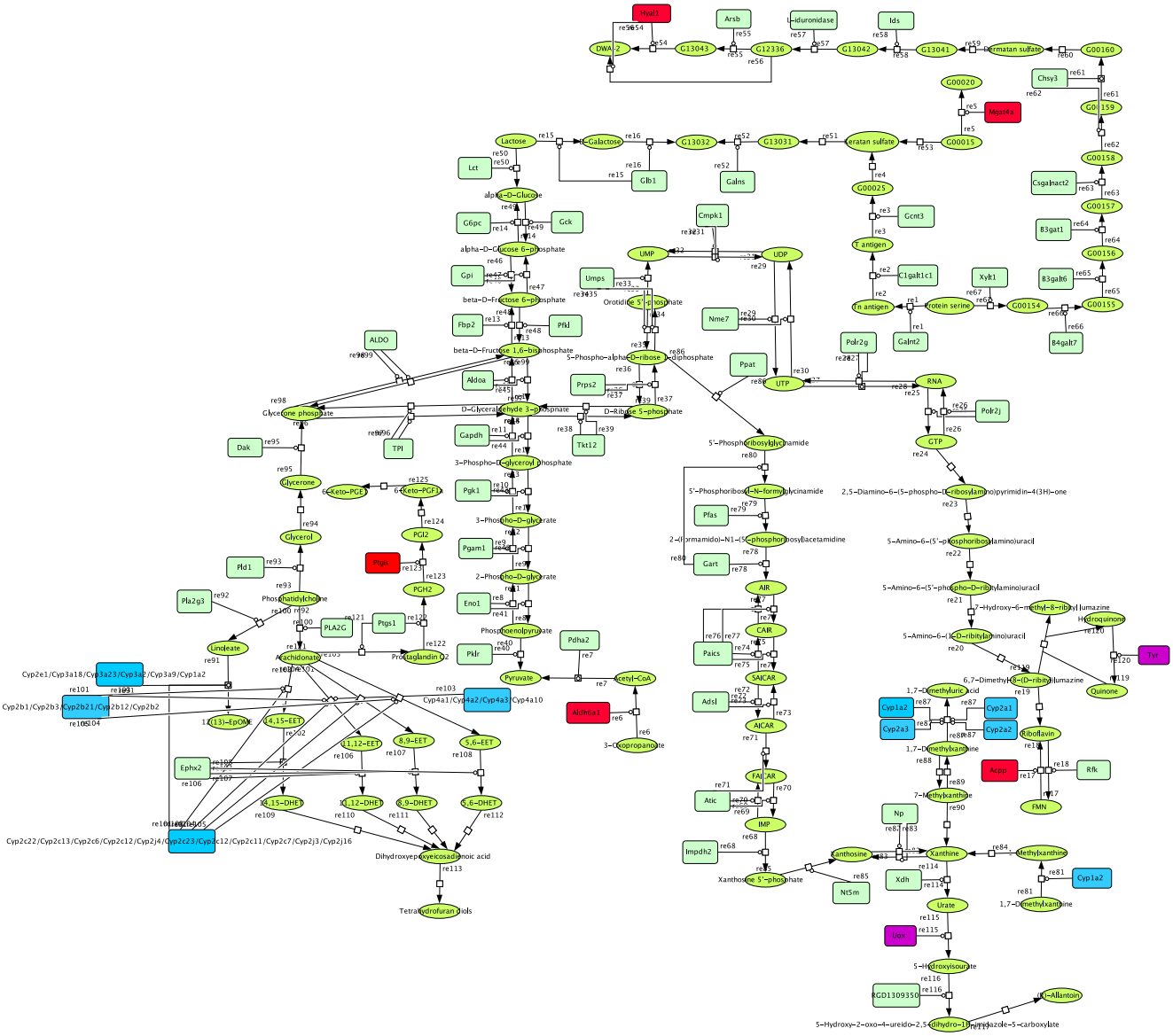

Fig. 3 The connected pathways related to the genes identified directly in the differential analysis (in *red*), to the p450 family (in *blue*) and to urate oxidase (*Uox*), via the purine metabolism pathway and to tyrosinase (*Tyr*) through the riboflavin metabolism pathway (in *purple*). High-resolution file of this figure is available as Online Resource 2

Table 2 Lists of metabolites and the P450 superfamily isoforms for which they are substrates

Substrate	p450 Isoforms
Testosterone	3A4, 5, 7, and 43
Arachidonic acid	4A11, 4A22, 4B1, 4F2,3, 8 4F11, 4F12 4V2, 4X1, and 4Z1
Thromboxane A_2 synthase	5A1
Bile acid	7A1 and 7B1
Prostacyclin synthase	8A1
Vitamin D	24A1
Retinoic acid hydroxylase	26A1, 26B1, and 26C1
Linoleate	*2E1, 1A2, 2A2, 9, 18, and 23*
Dymetil-xantine	*1A1 and 2,3*
Cafeine	*1A2*
Arachidonate (anion of arachidonic acid)	*2B1, 3, 12, and 21; 4A1, 2, 3, and 10; 2C6, 7, 11, 12, 13, and 22; 2J3, 4, and 16*
cholesterol	51A1

Substrates set in *italics* are the ones highlighted as relevant in this analysis

additional possible metabolic targets to be included in future devices for continuous monitoring of cell cultures, based on biosensors for uric acid [41] and glutamine and glutamate [42].

4 Conclusions

Our gene expression analysis showed that the tight and multidisciplinary interaction among molecular biologists, bionformaticians, and engineers, following a multi-step pipeline based on a variety of tools, can generate original findings that become the input for the generation of novel information first, and of novel technology then, in a continuous and virtuous spiral of improvement.

The example we have shown outputs a set of metabolites that can be considered to develop a completely new low-throughput biochip for continuous monitoring (with an extremely fine time scale) stem cell cultures at the single metabolite level. Therefore, the synergy between the high-throughput techniques with the low-throughput one, became the next frontier for previously un-imaginable investigations. Despite currently being limited to a single biological system, the approach we are proposing can be extended to a number of systems, and it represents a proof of principle of the feasibility of such approach. Nevertheless, we understand that number of obstacles are still to be overcome in moving from the identification of the sensing and sensed variables to the actual chip implementation. However, we expect such type of work to become more and more popular, given the growing number of high-throughput screens that are being made available at lower costs [43] and the consequent more precise approaches and tools that can be developed [44] to get more insight into the molecular complexity shown by these screens.

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