Reprogramming of fish somatic cells for nuclear transfer is primed by Xenopus egg extract

Nathalie Chênais, Aurélie Le Cam, Brigitte Guillet, Jean-Jacques Lareyre, Catherine Labbé

1   **Reprogramming of fish somatic cells for nuclear transfer is primed by *Xenopus* egg extract**

2

3   **AUTHOR LIST AND AFFILIATIONS**

4   Nathalie Chênais[1]*, Aurelie Le Cam[1], Brigitte Guillet[2], Jean-Jacques Lareyre[1], Catherine Labbé[1]*

5   [1] INRAE, UR1037 LPGP, Fish Physiology and Genomics, Campus de Beaulieu, F-35000 Rennes, France

6   [2]Université de Rennes 1, Campus de Beaulieu, F-35000 Rennes, France

7

8   ***CORRESPONDING AUTHORS**

9   Nathalie Chênais: nathalie.chenais@inrae.fr

10   Catherine Labbé : catherine.labbe@inrae.fr

11

12   **ABSTRACT**

13   Somatic cell reprogramming *in vitro* prior to nuclear transfer is one strategy expected to improve clone

14   survival during development. In this study, we investigated the reprogramming extent of fish fin

15   somatic cells after *in vitro* exposure to *Xenopus* egg extract and subsequent culture. Using a cDNA

16   microarray approach, we observed drastic changes in the gene expression profile of the treated cells.

17   Several actors of the TGFβ and Wnt/β-catenin signaling pathways, as well as some mesenchymal

18   markers, were inhibited in treated cells, while several epithelial markers were upregulated. This was

19   associated with morphological changes of the cells in culture, suggesting that egg extract drove

20   somatic cells towards a mesenchymal-epithelial transition (MET), the hallmark of somatic

21   reprogramming in induced pluripotent stem cells (iPSCs). However, treated cells were also

22   characterized by a strong decrease in *de novo* lipid biosynthesis metabolism, the lack of re-expression

23   of *pou2* and *nanog* pluripotency markers, and absence of DNA methylation remodeling of their

24   promoter region. In all, this study showed that *Xenopus* egg extract treatment initiated an *in vitro*

25   reprogramming of fin somatic cells in culture. Although not thorough, the induced changes have

26   primed the somatic chromatin for a better embryonic reprogramming upon nuclear transfer.

27

28

29   **INTRODUCTION**

30

31   In fish, somatic cells and particularly fin cells are a convenient source of diploid material for

32   cryopreservation of valuable genetic resources [1]. Such use of somatic cells compensates for the

33   impossibility to cryopreserve fish oocytes and embryos. Besides, fin cells are easy to collect whatever

34   the sex, maturation status or size of the fish, and they are easy to cryopreserve [2,3]. However,

35   regeneration of fish from these highly differentiated cells requires to master nuclear transfer, a

36   technology that is still not reliable enough in fish. Indeed, whereas nuclear transfer with embryonic

37   donor cells yields acceptable development rates [4–8], only few clones were reported to reach

38   adulthood when the donor cell was taken from adult fish [9–16]. One hypothesis often proposed to

39   explain the low success rate of somatic cell nuclear transfer is the chromatin reprogramming failure

40   (reviewed in mammals [17]). In fish, zebrafish clones at dome stage fail to re-express several genes that

41   are important for chromatin remodeling, translation initiation or cell cycle[18]. More recently, we

42   showed that DNA methylation of several marker genes in goldfish clones failed to match the

43   hypomethylation status of control embryos, and some clones bore the hypermethylated pattern of the

44   donor fin cells [19]. This means that after nuclear transfer, exposure of the somatic chromatin to oocyte

45   factors prior to embryonic genome activation is not sufficient to overcome somatic cell resistance to

46   reprogramming in fish.

47

48   Numerous studies in mammals have sought to improve the reprograming ability of donor somatic cells

49   by way of an *in vitro* pre-reprogramming before nuclear transfer. The ability of metaphase-II (MII) egg

50   factors to ensure chromatin remodeling of sperm and oocyte chromatin following fertilization makes

51   the egg extract an attractive candidate for *in vitro* reprogramming. Heterologous *Xenopus* eggs at MII

52   stage have been reported to improve the blastocyst rates after nuclear transfer in mouse [20], ovine [21]

53   and porcine [22,23], and although assessed in only few studies, it also increased the development success

54   after implantation or birth [21]. At the molecular level, these heterologous egg extracts have also been

55   shown to induce transcriptional and epigenetic remodeling in mammalian cultured cells. However,

56   such reprogramming of somatic cultured cells is not straightforward and it suffers high variability.

57   Many factors such as the animal species and cell type [24,25], the culture conditions and egg extract

58   batches or stages [26,25,27,20] influenced the extent of somatic cell reprogramming. For example, when

59   considering the expression of pluripotency markers, porcine cells treated with *Xenopus* egg extract

60   only transiently re-expressed *Oct4* over culture time [26,28] and *Nanog* expression failed to be

61   consistently re-expressed [26,29] while in mouse, *Oct4* and *Nanog* were both re-expressed [30,20].

62   Moreover, the extent of reprogramming at the scale of the whole genome is not known. Indeed, all

63   studies are assessing the reprogramming success from candidate genes analysis, and a reprogramming

64   assessment based on all other putative actors of pluripotency is still missing. As a consequence,

65   knowledge on the gene network rewiring upon *in vitro* reprograming with egg extracts remains elusive.

66

67   The question is still open in fish as to whether *Xenopus* egg extract can alter the course of somatic cells

68   in culture, and if this treatment bears the potential to later on improve nuclear transfer in fish species.

69   In a previous study, we demonstrated that goldfish fin cells in primary culture can incorporate *Xenopus*

70   MII-egg extract molecules such as LaminB3 in their nucleus [31], thus providing evidence that egg factors

71   can reach somatic cells chromatin. It has been described in mammals that the reprogramming effect

72   of *xenopus* egg extracts requires a culture step of the cells, so that they can recover from the treatment

73   and that the new cellular program can induce changes in gene expression [26]. However, the treated

74   cells in our former work were too fragile to be cultured, and the reprogramming consequences of the

75   treatment had been impossible to study. In the present work, we have set up a procedure which

76   allowed the survival and proliferation of the goldfish treated cells in culture. This enabled the analysis

77   of their reprogramming extent. The use of nuclear transfer success as a mean to assess the extent of

78   donor cell reprograming was excluded because of the multiparametric factors at stake in clone

79   development success in fish. Indeed, embryonic failures are a combination of mitotic errors [32] and

80   gene reprogramming defects [18,16] whose respective contribution is highly variable between clones.

81   Therefore, the consequences are impossible to discriminate one from the other at the embryonic

82   genome activation stage, when most clones fail to develop [11–13].

83

84   The aim of the present work was to investigate the response of goldfish somatic cells to treatment

85   with *Xenopus* egg extract in culture, and to assess whether this treatment triggered some

86   reprogramming events that would take place ahead of cell collection for nuclear transfer. Changes in

87   gene expression were analyzed by an unbiased microarray approach, and specific networks associated

88   with reprogramming were sought, in relation with the behavioral changes of the cultured cells. At the

89   epigenetic level, changes in DNA methylation pattern of some candidate genes were also explored,

90   namely the *pou2* and *nanog* genes that have differentially methylated promoters between fin cells and

91   embryonic cells [33,34]. Cells from primary fin culture were chosen over cell lines, because the former

92   are closer to the original genetic background that is sought for regeneration of valuable fish genotypes

93   by nuclear transfer.

94

95

96

97   RESULTS

98

99   Validation of fin cell exposure to *Xenopus* egg extract

100

101   The mesenchymal cell preparation and treatment that were set up in a previous study [31] included

102   plasma membrane permeabilization with digitonin, permeabilized cell exposure to egg extract for 1h,

103   and plasma membrane resealing (Supplementary Fig. S1). Penetration of egg factors per se was not

104   tested here, because this would have required cell fixation. However, all cells displayed the phenotypic

105   characteristics of permeabilized cells and egg extract-treated cells that were described previously [31]:

3

106   their nuclear membrane was more contrasted after permeabilization than in control cells, their

107   adhesion capacity lessened during egg extract exposure and remained very low during the resealing

108   step and the first 24 h of culture, and they all adopted a round and refracting morphology after

109   resealing. Taken together, our observations indicate that all treated cell batches in the present study

110   did incorporate egg extract.

111

112   Treated cells need a suitable culture medium to survive and proliferate.

113

114   The culture phase of the treated cells had to be mastered, so that the treated cells could undertake

115   their new cellular program. When the conventional L15 medium was used, we observed that from the

116   second day of culture on, many treated cells displayed a cubic shape (Fig 1, left picture) that contrasted

117   with the much more elongated control cells (Fig 1, central picture). However, after 7 days of culture in

118   L15, the treated cell density decreased, and many cells detached from the culture plate (Fig 1, left

119   pictures), whereas control cells kept proliferating (Fig 1, inset d7). This inability of the treated cells to

120   survive in L15 medium provided a first indication that the egg extract treatment had induced some

121   changes in the treated cell physiology.

122

123   Therefore, we sought for a culture medium that would sustain treated cells survival and proliferation,

124   while maintaining their modified state. In mammals, somatic cells treated with egg extract were

125   reported to be cultured in embryonic stem (ES) cells medium containing Leukemia Inhibitory Factor

126   (LIF) and other complements aimed to prevent cell differentiation [29,25,27]. However, fish ES-like cells

127   are known to be independent from LIF (reviewed in [35]). Furthermore, the maintenance of an

128   undifferentiated state in zebrafish and medaka ES-like cultured cells was reported to require a medium

129   enriched with fish serum and species-specific embryo extracts, known as ESM4 medium [35,36]. We

130   therefore tested the ESM4 medium enriched with goldfish embryo extracts (supplementary Table S1).

131   After 2 days in this new culture medium, the cubic shape of the treated cells seen in L15 was

132   maintained in ESM4 (Fig 1, right pictures). The elongated shape of the control cells was not changed

133   either, indicating that ESM4 has no effect of its own on the shape of the cultured cells. Most

134   interestingly, the treated cells cultured in ESM4 were able to proliferate over longer culture time

135   compared to culture in L15 medium. They showed an increased cell density at day 7, and debris and

136   floating cells were no longer observed (Fig 1, right picture). Additionally, they maintained their specific

137   cubic morphology. This demonstrates further the favorable effect of ESM4 medium on the growth of

138   the modified treated cells.

139

140   Changes in gene expression eight days after egg extract treatment.

4

141   *Clustering of the differentially expressed genes (DEGs)*

142   Analysis of the microarray data revealed that 2,286 goldfish genes out of the 52,362 genes on the

143   microarray were differentially expressed between treated and control cells (fold change > 2).

144   Additionally, hierarchical clustering analysis of the differentially expressed genes (DEGs) showed a

145   clear segregation between treated and control samples (Fig 2, upper dendrogram). This demonstrated

146   that the treated cells transcriptome was modified by egg extract treatment and that the consequences

147   were detectable after 8 days of culture. Differentially expressed genes between treated and control

148   cells showed a distribution into two clusters on the heatmap. Cluster I gathers 872 genes

149   (encompassing 38 % of the DEG) that showed upregulation in the treated cultured cells. Cluster II

150   comprises 1 414 genes (62 % of the DEG) that were down regulated in the treated cells. Genes in each

151   cluster are listed in Supplementary Table S2.

152

153   *Segregation of the treated samples according to egg-extract batches.*

154   Although all treated samples segregated together and showed the same expression profile clustering,

155   it is noteworthy that samples T1 to T3 segregated together, apart from the 4 other samples (T4-T7)

156   (Fig 2, upper dendrogram). One possible explanation lies in the egg-extract batches that were used for

157   the different samples. Indeed, the extracts were all prepared from freshly spawned MII stage eggs,

158   each extract being obtained from the spawn of a different female. We cannot exclude that the

159   individual extracts presented some quality variations one from another, notably because of the

160   instability of MII stage in spawned eggs [37]. In order to validate the egg extract stage, we used two MII

161   markers: Greatwall whose phosphorylated forms prevent mitosis/meiosis exit [38], and Cyclin B whose

162   degradation characterizes mitosis/meiosis exit. Egg extracts arrested at MII stage all displayed a

163   specific western blot profile (supplementary Fig. S2): Greatwall (Gwl) was phosphorylated and stable

164   over incubation time, and cyclin B (CycB) content was high and stable as well. Upon in vitro induction

165   of MII exit by Ca^{2+}, Greatwall was successfully dephosphorylated and cyclin B underwent degradation.

166   Contrarily to these well-defined MII egg extracts, some egg extracts showed Greatwall

167   dephosphorylation and Cyclin B degradation, indicating that they had initiated MII exit (MII late stage).

168   Interestingly, the egg extracts used to treat T1 to T3 samples where in MII stage whereas those of T4

169   to T7 samples had initiated MII exit to some extent (Supplementary Fig. S2). As a conclusion, the

170   sample segregation in the treated cells was likely related to the extract stages (MII and MII-late). This

171   highlights the importance of a careful characterization of the *Xenopus* egg extracts. Although the

172   sample number in each category was low, we still performed a fold change analysis between the two

173   groups. We observed that 83% of the DEGs between MII and MII-late extract groups had low fold

174   changes (< 6), and only 52 genes had fold changes above 6, among which only 9 genes were above 20.

175   Besides, no significant or straightforward biological processes were identified via the GO terms analysis,

176 and no marker gene of any specific biological significance emerged from a gene to gene scouting. To

177 conclude, and within the limits of this small sampling, the egg extract stage did not thoroughly affect

178 cellular response, and the two clusters of up- and downregulated genes were observed in all 7 treated

179 cells batches irrespective of the egg extract that was used.

180

181 *Gene Ontology (GO) analysis of the differentially expressed genes after egg extract treatment.*

182 GO analysis was a perquisite in order to process our DEG list into functions and biological significance.

183 For this purpose, we had first to translate the goldfish gene identifiers into those of the closest species

184 whose genome is well annotated in the GO databases, the zebrafish. This artificially reduced the

185 number of DEG, because the zebrafish did not undergo the genome duplication reported in the

186 *Cyprininae* sub-family to which goldfish belongs. Only 1533 zebrafish genes (i.e. 67% of total goldfish

187 DEG) were retained for subsequent annotations in GO. Of these, 591 annotated genes were up-

188 regulated (cluster I) and 942 annotated genes were down regulated (cluster II) in the treated cells. GO

189 analysis conducted with the WebGestalt web tool [39] showed that biological regulation and metabolic

190 process were the most represented terms (supplementary Table S3) among biological process GO

191 terms. Surprisingly, no straightforward reprogramming processes such as chromatin remodeling, stem

192 cells, transcription factors, or pluripotency could be emphasized in GO terms after a statistical over-

193 representation analysis. However, several other biological process terms significantly enriched in the

194 GO terms list deserve specific attention

195

196 Deregulation of TGFβ and Wnt signaling pathways after egg extract treatment.

197

198 Our work is reporting gene expression variation, but GO databases and related publications on gene

199 function report mainly protein functions. It is therefore the protein writing nomenclature that will be

200 used in the following sections dedicated to GO interpretation. The most significant GO term obtained

201 from the cluster of up-regulated genes is the cell surface receptor signaling pathway (Fig 3A). The data

202 mapping showed that this GO term was linked to highly significant child GO terms that are

203 transforming growth factor beta (TGFβ) receptor signaling pathway, and Wnt signaling pathway

204 together with regulation of canonical Wnt signaling pathway. This result was consistent with the KEGG

205 (Kyoto Encyclopedia of Genes and Genomes) analysis performed on the same set of DEG data, that

206 also showed that both TGFβ and Wnt signaling pathways reached a significant level of enrichment

207 among all the database terms (Fig 3B).To add on to the highlighting of these 2 specific pathways, we

208 also observed an enrichment in the GO terms related to the MAPK / ERK cascade (Fig 3A), known to be

209 one of the non-canonical pathways activated by TGFβ [40]. Thus, the GO analysis based on the cluster

6

210   of up-regulated genes clearly highlighted the TGFβ and Wnt signaling pathways as major ones being

211   affected in the cells exposed to egg extract reprogramming factors.

212

213   *TGFβ signaling*

214   TGFβ signaling is involved in numerous biological processes related to embryonic development. We

215   then checked the actors of the TGFβ pathway present in our goldfish DEG list (irrespective of their up

216   or down regulation). TGFβ belongs to the superfamily of the growth factors, divided into several

217   subfamilies including TGFβs, and Bone Morphogenetic Proteins (BMPs). For TGFβ signal to be

218   transduced, the TGFβ ligand binds type II receptors. Ligand - type II receptor complex triggers the

219   recruitment of TGFβ type I receptor, and the dimerized receptors subsequently activates specific Smad

220   proteins, able to induce transcription of the TGFβ target genes [41,42]. Beyond signaling pathways

221   involving Smads, known as canonical TGFβ pathways, other pathways independent of Smads are also

222   controlled by TGFβ, including the MAPK Erk1 / ERk2 pathway identified above by GO analysis (Fig. 3A).

223   We therefore analyzed the expression profile of these TGFβ actors and their biological partners.

224

225   We found that some TGFβ and BMP ligands together with type I receptors were upregulated in treated

226   cells compared to controls (Table 1, TGFβ Effectors). However, this upregulation is unlikely stimulating

227   the TGFβ signaling pathway, because the key actors binding TGFβ that are the TGFβ type II receptors

228   did not change their expression pattern in treated cells. Besides, most other actors of the TGFβ

229   signaling pathway identified in this study were affected in the direction of a TGFβ signaling inhibition

230   in the treated cells, namely inhibitors upstream of TGFβ signaling that were upregulated in the treated

231   cells (Table 1, TGFβ Inhibitors). Among them, we identified extracellular inhibitors (lft2, nog1, nog2,

232   grem2a, grem2b) and membrane inhibitors (Bambia and Bambib) that are binding to TGFβ and BMP

233   ligands. Such binding prevents TGFβ and BMP to attach to their own receptors, thereby preventing

234   signal transduction activity [43,44]. Beyond these inhibitors, we also found intracellular inhibitors

235   (involved in TGFβ canonical signaling pathway) which included specific smads (smad6a, smad6b,

236   smad7, smad9) and the ubiquitin ligase smurf2 (Table 1). The combined action of Smad7 and Smurf 2

237   is known to induce TGFβ type I receptor degradation by the proteasome [42,45], leading to inhibition of

238   the TGFβ canonical pathway. Finally, spry1, sry4, and dusp6 genes, inhibiting the MAPK / ERK pathway

239   (non-canonical TGFβ pathway), were also found upregulated in the treated cells (Table 1). In all, our

240   gene to gene analysis of the expressional changes of TGFβ actors, including the non-canonical MAPK /

241   ERK pathway, indicated that fin cells exposure to egg extract induced an overall inhibition of the TGFβ

242   signaling pathway.

243

244   *Mesenchymal-epithelial transition*

7

245   In mammals, one consequence of TGFβ signaling inhibition is the induction of a mesenchymal-

246   epithelial transition (MET), considered to be a hallmark of iPSC early phase reprogramming, and

247   described as crucial for reaching pluripotency [46–50]. MET is characterized by the loss of mesenchymal

248   markers and by the activation of genes determining epithelial fate [47]. We therefore investigated

249   whether inhibition of TGFβ signaling in our fish treated cells was also associated with changes in MET

250   marker genes. We found that many mesenchymal marker genes were downregulated, among which

251   several members of the collagen family, matrix-metallo protease (mmp9) and fibronectin (fn1) (Table

252   2). The fn1 gene was the most strongly affected (- 44 fold change). This was associated with the

253   concomitant upregulation of several epithelial marker genes such as cadherins (pcdh1 cadherin-like 1,

254   pcdh12, cdh18, cdh24b), cytokeratins (krt15, krt18), and cell junction proteins such as pkp3b, cldn5a,

255   tjp1a and cx43 (Table 2). Regarding the gap junction component cx43, it is known to be specifically

256   enriched in epithelial cells and iPSCs, and its ectopic expression and gene upregulation has been

257   associated with an increase in reprogramming efficiency by facilitating MET [51]. Last, the transcription

258   factor zeb1 known to induce EMT (epithelial-mesenchymal transition) [52], ie the reverse of the MET,

259   was downregulated in treated cells (Table 2). In all, the observed expressional changes suggest the

260   initiation of a MET program in the treated cells. This expressional profile is in accordance with the

261   epithelial-like morphology reported above for the treated cells, which were more cubic than the

262   elongated control cells. However, we observed from this DEG analysis and from qPCR analysis that one

263   abundant mesenchymal marker, col1a1a, remained highly expressed in treated cells and was not

264   differentially expressed between the two conditions (relative expression 125.0 ± 52.8 in treated cells,

265   n=7 ; 123.1 ± 25.8 in control cells, n=8). This suggests that MET would be initiated but not terminated

266   in our culture conditions.

267

268   *Wnt signaling*

269   The second signaling pathway whose terms were enriched in the GO analysis is the Wnt signaling

270   pathway, and particularly the canonical one (Fig. 3). Because β-catenin is a key effector of Wnt signaling,

271   the canonical pathway is referred to as Wnt/β-catenin signaling. The transduction of Wnt signal

272   requires Wnt-induced activation of the receptors complex made of Frizzled (fzd) receptor and low-

273   density lipoprotein co-receptor related 5 or 6 (LRP5/6). In other words, binding of the Wnt ligand to

274   both receptors creates and activates the receptors complex. This initiates a series of molecular events

275   that will protect cytosolic β-catenin from degradation. After nuclear import, β-catenin subsequently

276   triggers the transcription of Wnt target genes by binding to transcription factors belonging to the T-

277   cell factor/Lymphoid enhancer factor (Tcf/Lef) family [53,54].

278

279 Our gene to gene analysis of these Wnt-related actors revealed a strong deregulation of the Wnt/β-
280 catenin signaling pathway in egg-extract treated cells (Table 3). Up-regulation of Wnt effectors
281 combined with down- and up-regulation of inhibitors prevented the identification of a straightforward
282 status for the Wnt signaling, be it an activated "on" or inhibited "off" status. In favor of an "on" status
283 is the fact that some secreted Wnt ligands and fzd receptors were up-regulated in treated cells, the
284 expression of the fzd10 receptor being especially strong. Moreover, extracellular Wnt agonists R-
285 spondins (rspo2, rspo3), known to increase fzd receptors availability on the cell surface [53] and to
286 stabilize the LRP5/6 co-receptors [54], were up regulated in treated cells. Additionally, down regulation
287 of the extracellular inhibitors sfrp1a, sfrp2 and dkk1a [53] should be inducing a better availability of the
288 Wnt ligand for fzd receptors.

289

290 However, other observations would be more in favor of an "off" status of the Wnt signaling. Firstly,
291 the co-receptors LRP5/6 expression was not changed by the treatment. Despite increase in Wnt ligands
292 and fzd receptors gene expression, LRP5/6 stability would stoichiometrically hamper the formation of
293 the ternary proteic complex Wnt ligand / fzd receptor / LRP5/6 co-receptors that is essential for signal
294 transduction. Secondly, many extracellular inhibitors upstream of the signaling pathway were
295 upregulated in treated cells (Table 3). These included (i) notum1a and frzb, known to prevent Wnt
296 ligand from binding to fzd receptor [54,55], (ii) sclerostin (sost) and dkk1b, which are blocking Wnt-fzd-
297 LRP5/6 complex formation by interacting with LRP5/6 [53] and, (iii) kremen1, a membrane receptor
298 which interacts with dkk1 to increase the removal of the LRP5/6 co-receptors from the cell surface by
299 endocytosis [56]. Finally, the last point concerns the Tcf/Lef transcription factors, known as Tcf1, Tcf3,
300 Tcf4 and Lef1 in mammals, which control the Wnt signaling activity through transcription of the target
301 genes. In this study, Tcf7 (orthologue of Tcf1 in mice) expression was upregulated in treated cells (Table
302 3). It is known that the action of Tcf1 is triggered by nuclear beta catenin levels (Grainger 2019). Tcf1
303 acts as a transcriptional activator of Wnt target genes in presence of beta catenin. Conversely, Tcf1
304 acts as a transcriptional repressor of Wnt signaling in absence of beta catenin (Grainger 2019). In our
305 study, treated cells showed a considerable collapse of fn1 and to a lesser extent a downregulation of
306 pak1, also known as p21 (Table 3). The downregulation of these Wnt target genes associated with Tcf7
307 upregulation suggest that Tcf7 would act rather as a repressor in treated cells due to low beta catenin
308 levels. All these observations reinforce the hypothesis that the deregulation of the Wnt signaling in
309 treated cells would be rather in "off" configuration.

310

311 *Altered cell adhesion of the treated cells and link with the deregulation of TGFβ and Wnt signaling*
312 *pathways*

313   In addition to the change in treated cells morphology, we also reported above a change in their

314   behavior in culture. The cells showed a highly reduced ability to adhere throughout the culture process,

315   and this could be due to changes in some gene expression. And indeed, from the GO analysis of all

316   DEGs between treated and control cells, one biological process GO term highlighted the cell adhesion

317   process (GO: 0007155; *P value*=3.2560E-08; FDR=1.17E-05). Furthermore, fibronectin (fn1) is a major

318   protein of the extracellular matrix and it provides highly adhesive capacity to the cells by interaction

319   with integrin transmembrane receptors [57]. As shown above, this actor was one of the most highly

320   downregulated gene in our conditions, and this reduced expression is known to be deeply

321   interconnected with the observed inhibition of the TGFβ and Wnt signaling pathways (Table 2, Table

322   3) [58]. In all, the poor adhesion capacity of our treated cells is another indication of the undergoing

323   reprogramming event triggered by changes in the TGFβ and Wnt signaling pathways expression.

324

325   Some pluripotency markers remained silent in the treated cells

326

327   The process of somatic reprogramming in iPSCs is generally encompassing two phases [59]: (i) an early

328   or initiation phase during which the somatic cells undergo a MET, lose their mesenchymal

329   characteristics and develop an epithelial phenotype and, (ii) a late maturation phase allowing the

330   reactivation of the pluripotency network. In order to characterize further the reprogramming extent

331   of the treated fin cells, we focused on some marker genes related to pluripotency, previously

332   characterized in goldfish during early development: *pou2* (*pou5f3* in zebrafish, *oct4* in mammals),

333   *nanog*, *sox2* and *c-myc* [33,34,60]. We observed that none of these genes were identified among the DEGs,

334   and their expression levels remained undetectable on the microarray. These observations were

335   confirmed by qPCR that showed that *pou2*, *nanog*, *sox2* and *c-myc* expression was below detection

336   threshold in both treated and control cells.

337

338   It was shown previously in goldfish that *nanog* and *pou2* silenced status in fin cells is associated with

339   the hypermethylation of a CpGs locus in their promoter region [33,34]. We also showed recently that

340   after nuclear transfer with non-treated fin cells, these loci underwent a partial and stochastic

341   demethylation in the developing clones [16]. This indicated that embryonic reprogramming relaxed the

342   DNA methylation status of these marker regions to some extent. The methylation profile of *nanog* and

343   *pou2* promoter regions in our treated cells was therefore analyzed, to assess whether some DNA

344   demethylation took place at these marker sites after *xenopus* egg treatment. This would indeed be a

345   necessary step ahead of any transcription reenabling of these 2 genes. Analysis of the CpG sites in *pou2*

346   and *nanog* promoter regions revealed that they did not underwent any significant demethylation in

347   treated cells (Supplementary Fig. S3). Although the methylation of some CpG sites was lower in treated

10

348   cells compared to controls, there was no significant differences in the overall DNA methylation rate of

349   *pou2* and *nanog* promoter regions. This indicates that no significant remodeling of the DNA

350   methylation of *pou2* and *nanog* pluripotency genes was triggered following egg-extract treatment,

351   even if some minor variations were observed. In all, the silenced status of pluripotency marker genes

352   associated with the absence of significant DNA methylation remodeling support the idea that treated

353   cells would have been only partially reprogrammed by *Xenopus* egg-extract treatment. Our cells would

354   not have reached the maturation phase of reprogramming characterized by Oct4 or Nanog and Sox2

355   re-expression as observed in mammalian somatic cells.

356

357   Alteration of *de novo* lipid biosynthesis in response to egg-extract treatment

358

359   Regarding the cluster of downregulated genes, the GO biological processes the most significantly

360   affected by egg-extract are related to lipid metabolism (Fig 4A). Child GO terms targeted biosynthesis

361   of steroid including cholesterol, and biosynthesis of unsaturated fatty acid. This was consistent with

362   KEGG analysis showing the enrichment of the biosynthesis pathways of steroids, unsaturated fatty

363   acids as well as the pathway of fatty acid metabolism (Fig 4B). In this process, acetyl-CoA represents

364   the main precursor for de novo lipid biosynthesis. Produced in the mitochondria after glycolysis, acetyl-

365   coA has to be metabolized into citrate so that it can exit the mitochondria. Once in the cytoplasm,

366   citrate is then converted into lipogenic acetyl-CoA (see the molecular actors of lipogenesis in [61]). A

367   detailed analysis of lipid metabolism genes showed a downregulation of several genes involved in the

368   cytosolic synthesis of acetyl-CoA i.e. slc25a1b, a key mitochondrial transporter of citrate, aclya, which

369   converts cytoplasmic citrate to acetyl-CoA, acss2, which produces acetyl-CoA from acetate, and the

370   acyl transferase acat2 (Table 4). Lipid biosynthesis is also controlled by srebf1/2 transcription factors,

371   whose expression was downregulated in our treated cells. The target genes of these transcription

372   factors were downregulated as well. These included key enzymes for biosynthesis of cholesterol

373   (hmgcs1, hmgcra1, msmo1, fdft1, cyp51, dhcr7) and fatty acid (fasn, sdc, elov1a, elov2, elov5, elov6)

374   (Table 4). Overall, our results clearly indicate that the treated cells have strongly reduced their *de novo*

375   lipid biosynthesis compared to control cells.

376

377   **DISCUSSION**

378   In this work, we explored to what extent fin somatic cells in culture could be modified by exposure to

379   *Xenopus* egg-extracts. Our objective was to relax these differentiated cells so that they would be better

380   fitted for reprogramming after nuclear transfer in fish. We showed that the treatment triggered some

381   phenotypic changes of the cells, namely reduced adhesion capacity, adoption of a cubic shape

382   morphology (epithelial-like), and reliance on the ESM4 medium for survival and proliferation. At the

383   transcriptomic level, the thorough coverage of all goldfish transcripts and the number of biological

384   replicates in our microarray analysis allowed us to identify two clusters of differentially expressed

385   genes. We showed by GO analysis that cell surface receptor signaling pathways and lipid metabolism

386   were the most significant terms that stood out from the list of these genes. Actors of the TGFβ and

387   Wnt/β-catenin signaling pathways were altered in a pattern indicative of their inhibition, and this was

388   combined with markers of a MET initiation. These changes were also associated with the lack of

389   restoration of pluripotent markers activity and of their promoter demethylation, and with a reduction

390   of lipid biosynthesis.

391

392   Evidences of a reprogramming priming of the cells treated by *xenopus* egg extract

393

394   Reprogramming of somatic cells into a less differentiated state encompasses a series of molecular

395   changes whose sequence includes the downregulation of somatic markers and of some signaling

396   networks, induction of MET, and activation of early pluripotency markers [62,63]. The TGFβ signaling

397   inhibition observed in our treated cells, including that of the non-canonical MAPK / ERK pathway,

398   would be one preliminary step in this reprogramming process. Indeed, experimental inhibition of TGFβ

399   signaling was shown to cooperate in the reprogramming of murine fibroblasts into iPSCs [46,49,64].

400   Furthermore, ERK inhibition was also shown to be an early molecular signature of somatic cell

401   reprogramming in this model species [65], and inhibition of both TGFbeta receptors and ERK [48] also

402   improved fibroblast reprogramming [66]. Beyond these examples, our treated cells also displayed

403   numerous MET markers, including the decreased expression of extracellular matrix genes, such

404   decrease being known to mediate fibroblast reprogramming [67]. Taken as a whole, our results lead us

405   to propose that the egg extract would have initiated a reprogramming of the fin cells by directing them

406   towards a MET via inhibition of TGFbeta signaling.

407

408   It is known that Wnt signaling deregulation is also triggering cellular reprogramming. In mice, it was

409   shown that Wnt off state matches the early phase of iPSCs reprogramming of embryonic fibroblast [68].

410   The off state of this signaling pathway observed in our study would indicate that our cells are in an

411   early stage of reprogramming. One notable consequence of Wnt off state is that among the

412   downstream target of the Wnt pathway, pak1 (orthologous to mammalian P21) was downregulated.

413   This senescence actor was shown to be a barrier to iPSCs reprograming [69,70] and to MET. We therefore

414   infer that its inhibition was favorable to MET and reprogramming in our treated cells.

415

416   Incomplete reprogramming of the treated cells

417

418   As explained above, the treatment applied to our culture cells was intended to increase somatic cell

419   plasticity towards reprogramming after nuclear transfer. Thus, our treatment with Xenopus egg

420   extracts remained within physiological limits, and it could not be expected to be as thorough as after

421   reprogramming into iPSCs. Several indicators in our study showed that indeed, the treated fin cells

422   were not entirely changed in their transcriptomic profile. Namely, although several collagens

423   underwent a reduced expression in the treated cells, col1a1a abundantly expressed in fin cells [71]

424   remained highly expressed after cell treatment. Furthermore, we failed to detect any re-expression of

425   the canonical pluripotency markers that are *pou2, sox2, nanog* and *c-myc*. This is at odd with the Oct4

426   re-expression induced with a similar treatment in porcine or human cultured cells [26,28]. Because of the

427   stochastic re-expression of these genes described by these authors and reviewed in [72], we infer that

428   we did not asses these markers expression in the right reprogramming window, or that our cells may

429   still be in the initiation or intermediate phase of reprogramming. This emphasize the importance of a

430   transcriptomic approach for a comprehensive assessment of cell reprogramming status.

431

432   Reduced lipid metabolism in the treated cells

433

434   We showed that the whole cluster of downregulated genes induced the high significance of GO terms

435   related to lipid metabolism, and numerous actors of the lipid biosynthesis were downregulated after

436   *Xenopus* egg treatment. This questions the role of lipids in our cellular reprogramming scheme. Indeed,

437   studies on iPSCs indicate on the contrary that increased lipid biosynthesis is favorable to MET and

438   reprogramming [73,74], and that conversely, inhibition of fatty acid biosynthesis blocks mouse embryonic

439   fibroblast reprogramming to iPSCs [75]. It was shown that large amounts of lipids are consumed during

440   the reprogramming process, as judged by the decreasing number of lipid droplets per cell between the

441   early and late stages of reprogramming [75]. This would indicate that lipid biosynthesis upregulation is

442   intended to provide for additional energetic resources to the cells undergoing reprogramming. This

443   hypothesis was explored in porcine iPSCs [76], and it was demonstrated that supplementation of the

444   culture medium with triglycerides, free fatty-acids, phospholipids and cholesterol improved the

445   reprogramming of embryonic fibroblasts by promoting MET.

446

447   Although we unambiguously showed in our study that egg-extract treatment failed to remodel the

448   lipid metabolism of fin cells according to iPSC pattern, we cannot exclude that lipid biosynthesis

449   downregulation was a response of the treated cells to the enriched ESM4 culture medium. In the

450   conventional L15 medium, the treated cells died after a few days. It means that if the treated cells

451   suffered endogenous lipid exhaustion, the lipids provided by their short-term exposure to *Xenopus* egg

13

452   extracts were not able to compensate for such losses. On the contrary, subsequent culture in the ESM4

453   medium containing extracts from goldfish embryos at 55 hours post fertilization stage may have

454   provided for the required energetic substrates. Fish embryos at this stage are indeed highly enriched

455   in cholesterol, phosphatidyl choline and triglycerides [77]. This means that ESM4 may have provided the

456   same MET-favorable environment as in [76]. In all, we may infer that the exogenous lipid supply via

457   ESM4 would have met the need of the treated cells, possibly supporting the MET requirements, and

458   that as a response, the lipid anabolism of the cells was reduced, leading to the observed

459   downregulation of the corresponding genes.

460

461

462   To conclude, the treatment of fish fin cells with *Xenopus* egg extract and subsequent culture in ESM4

463   induced phenotypical and expressional changes signing the induction of a reprogramming process via

464   MET. The identified reprogramming markers encompassed the TGFβ and Wnt/β-catenin signaling

465   pathways inhibition, the cubic cell shape, and the lessened cell adhesion capacity. We also provided

466   evidences that the reprogramming was obviously incomplete, as attested by the lack of pluripotency

467   markers re-expression, and maintenance of one abundant mesenchymal marker. Taken as a whole, it

468   appears that the fish somatic cells would be in early reprogramming phases, and that the treatment

469   helped to release some of the reprogramming barriers present in our differentiated fin cells. This

470   reprogramming priming by the *Xenopus* egg extract treatment could be a first favorable step towards

471   expression reprogramming and chromatin remodeling after nuclear transfer.

472

473   **METHODS**

474

475   Goldfish fin cell preparation

476

477   Two-year-old goldfish (*Carassius auratus*), 60 g mean weight, were obtained from outdoor ponds at

478   INRAE U3E experimental facility (Rennes, France), and maintained in recycled water at 14°C for several

479   weeks. Caudal fins were collected on euthanized fish following the French animal welfare guidelines

480   and under the French registration authorization n° 78-25 (N. Chênais). Fin cells were isolated and

481   cultured according to [31]. Briefly, fins were minced and digested with 2 mg/mL collagenase. Released

482   fin cells were plated in supplemented L15 culture medium. After 24 hours, adhering epithelial cells

483   were discarded while the supernatant enriched with slow adhering mesenchymal cells was collected.

484   These cells have previously been shown to be the most suitable for nuclear transfer [71,78]. After

485   filtration and washing, the mesenchymal cells were seeded at 0.2 x 10^6 cells per well in 24 well plates

486   and cultured in L15 medium for 2 days (about 80 % confluence) until *Xenopus* egg treatment.

487

488 *Xenopus* egg extract preparation and characterization

489

490 Unfertilized eggs from *Xenopus laevis* 2 years old females were obtained after hCG stimulation at the

491 CRB Xenope facility (University of Rennes 1, agreement number: 35–238-42). Egg extracts were

492 prepared as described previously [31]. Laid eggs were crushed at 10 600 g for 20 min at 4°C and the

493 extract was clarified at 10 600 g for 20 min at 4°C. The supernatant was collected, snap frozen in liquid

494 nitrogen and stored at -80°C. A total of 7 individual spawns were collected, providing 7 batches of

495 independent egg extracts with a protein concentration of 40 to 50 mg/mL and an osmolality of about

496 400 mOsm / kg.

497

498 Egg extract stage was characterized by western blot analysis using the mitotic markers Greatwall and

499 Cyclin B as described in [31]. For each egg extract, one fraction was immediately denatured in Laemmli

500 buffer at 95 °C (3 min). A second fraction was incubated at 25°C for up to 2 h prior to denaturation, to

501 mirror the time during which cells were treated with the egg extract. A last fraction was incubated with

502 0.8 mM Ca^{2+} at 25°C for up to 2 h to test its responsiveness to calcium-induced activation, before it

503 was denatured. MII status of the 7 egg extracts was determined using rabbit polyclonal *Xenopus* anti-

504 Greatwall and anti-Cyclin B (1:1000 each) according to [31].

505

506 Somatic cell treatment and culture.

507

508 Adherent mesenchymal cells were permeabilized with digitonin (30 ug/mL 2 min 4°C) before exposure

509 to egg extract (1 h, 25 °C), according to [31]. Cells were then incubated for 2 h in growth L15 medium

510 supplemented with 2 mM CaCl2 (25 °C) to reseal the plasma membranes and then cultured at 25°C in

511 ESM4 medium [35] (Supplementary Table S1). Culture medium was changed every 3 days. After 8 days,

512 cultured cells were collected after trypsinization and snap-frozen in liquid nitrogen. Non-permeabilized

513 cells were grown in L15 medium and used as controls. They followed the steps as the treated cells and

514 were snap-frozen after 8 days.

515

516 Microarray analysis

517

518 *Microarray preparation and hybridization*

519 Agilent 8x60K high-density oligonucleotide microarray (GEO platform no. GPL32340) was spotted with

520 a set of 52,362 distinct goldfish oligonucleotides. Available goldfish NCBI sequences were blasted on

521 the zebrafish genome, generating a list of zebrafish proteins identified as ENSDARP in the Ensembl

15

522 database. The official symbol of each gene, its description and its Ensembl ID, called ENSDARG, were

523 then extracted from the ENSDARPs using the Ensembl Biomart programm.

524

525 Total DNA and RNA of the cultured cells were extracted simultaneously after cell lysis in RNAsin (1 μL)

526 in Tri-Reagent, according the instructions for Miniprep DNA/RNA Direct-zol column extraction kit

527 (Zymo Research, R2081). RNA labeling and hybridization were performed according to the

528 manufacturer's instructions (Agilent "One-Color Microarray-Based Gene Expression Analysis (Low

529 Input Quick Amp labeling)"). For each sample, 150 ng total RNA was amplified and labeled using Cy3-

530 CTP. Yield (>825 ng cRNA) and specific activity (>6 pmol of Cy3 per μg of cRNA) of the obtained Cy3-

531 cRNA were checked on Nanodrop. Cy3-cRNA (600 ng) from each sample was fragmented, and samples

532 were hybridized on randomly chosen sub-arrays for 17 h at 65 °C. After microarray scanning (Agilent

533 DNA Microarray Scanner, Agilent Technologies, Massy, France), data were obtained with the Agilent

534 Feature Extraction software (10.7.3.1) according to the appropriate GE protocol (GE1_107_Sep09) and

535 imported into GeneSpring GX software (Agilent Technologies, Santa Clara, CA, USA) for analysis. Data

536 were published at the NCBI's Gene Expression Omnibus [79] and are accessible through GEO series

537 accession number GSE205854.

538 Of the 16 cell samples laid on the microarray, only 12 samples passed the quality controls and were

539 selected for analysis (n=5 control; n=7 treated with egg extract).

540

541 *Differentially expressed genes identification and Gene Ontology analysis*

542 The raw gene expression data were normalized and transformed into Log2 values using GeneSpring

543 software (Agilent). Only genes displaying an expression value significantly higher than that of the

544 background in at least 75% of the samples and in at least one of the two conditions were retained.

545 Selection of differentially expressed genes relied on a Student's t-test with false discovery rate (FDR)

546 correction and a fold change > 2 was applied. The significance level was set to FDR < 0.05 and p-value

547 < 0.05. The DEGs were then classified according to their expression profile by unsupervised hierarchical

548 clustering using Cluster3 software and were visualized by TreeView software.

549

550 A gene ontology analysis was carried out on the DEGs of each cluster using WebGestalt web tool

551 (AnaLysis web-based GEne SeT AnaLysis toolkit). In order to highlight the GO terms related to biological

552 process that were significantly enriched, an over-representation analysis (ORA) was carried out on the

553 gene IDs (ENSDARG) of each cluster. For this, each gene list of interest was compared to a background

554 gene list corresponding to all the genes spotted on the microarray. ORA was also carried out to search

555 for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. The significance level was set to

556 below an FDR 5 % and a p value of 0.05.

557

RTqPCR analysis

Confirmation of the expression pattern of several marker genes, namely *col1a1a*, *nanog*, *pou2*, *sox2*, *c-myca1* and *c-myca2*, were achieved by RTqPCR analysis according to [71]. The Ct values were normalized using the endogenous *18S rRNA* control and the target mRNA relative abundance was calculated according the formula: $2^{-\Delta Ct}$ with ΔCt = mean Ct (*target gene*) – mean Ct (*18S rRNA*).

DNA methylation analysis

Total extracted DNA was purified using the Genomic DNA Purification and Concentration Kit (Zymo Research, D4010) and quantified using the QubitTM dsDNA HS Assay Kit (Q32851, Invitrogen). DNA was treated with bisulfite using the EZ DNA Methylation-Gold kit (Zymo Research, D5006) and regions of interest were amplified according to [16]. Methylation status of the targeted CpGs was calculated after pyrosequencing with PyroMark Q24 ID 2.5 software (QIAGEN). Bisulfite conversion of control cytosines was above 98 %.

REFERENCES

1. Chenais, N., Depince, A., Le Bail, P.-Y. & Labbe, C. Fin cell cryopreservation and fish reconstruction by nuclear transfer stand as promising technologies for preservation of finfish genetic resources. *Aquacult Int* **22**, 63–76 (2014).

2. Mauger, P. E., Le Bail, P. Y. & Labbe, C. Cryobanking of fish somatic cells: optimizations of fin explant culture and fin cell cryopreservation. *Comp Biochem Physiol, Part B: Biochem Mol Biol* **144**, 29–37 (2006).

3. Moritz, C. & Labbe, C. Cryopreservation of goldfish fins and optimization for field scale cryobanking. *Cryobiology* **56**, 181–188 (2008).

4. Niwa, K., Ladygina, T., Kinoshita, M., Ozato, K. & Wakamatsu, Y. Transplantation of blastula nuclei to non-enucleated eggs in the medaka, Oryzias latipes. *Dev Growth Differ.* **41**, 163–172 (1999).

5. Wakamatsu, Y. *et al.* Fertile and diploid nuclear transplants derived from embryonic cells of a small laboratory fish, medaka (Oryzias latipes). *Proc Natl Acad Sci USA* **98**, 1071–1076 (2001).

6. Bubenshchikova, E. *et al.* Generation of fertile and diploid fish, medaka (Oryzias latipes), from nuclear transplantation of blastula and four-somite-stage embryonic cells into nonenucleated unfertilized eggs. *Clon Stem Cells* **7**, 255–264 (2005).

7. Tanaka, D., Takahashi, A. & Ueno, K. Morphometric characteristics and reproductive capacity of nuclear transplants derived from embryonic cells of loach, Misgurnus anguillicaudatus. *J Exp Zool, Part A: Ecol Genet Physiol* **311A**, 11–19 (2009).

8. Hattori, M., Hashimoto, H., Bubenshchikova, E. & Wakamatsu, Y. Nuclear Transfer of Embryonic Cell Nuclei to Non-enucleated Eggs in Zebrafish, Danio rerio. *Int J Biol Sci* **7**, 460–468 (2011).

9. Liu, T. M. *et al.* Factors affecting the efficiency of somatic cell nuclear transplantation in the fish embryo. *J Exp Zool* **293**, 719–725 (2002).

597 10. Lee, K. Y., Huang, H., Ju, B., Yang, Z. & Lin, S. Cloned zebrafish by nuclear transfer from long-term-
598   cultured cells. *Nat Biotech* **20**, 795–799 (2002).

599 11. Wakamatsu, Y. Novel method for the nuclear transfer of adult somatic cells in medaka fish (Oryzias
600   latipes): use of diploidized eggs as recipients. *Dev Growth Differ* 427–436 (2008).

601 12. Siripattarapravat, K., Pinmee, B., Venta, P. J., Chang, C. C. & Cibelli, J. B. Somatic cell nuclear
602   transfer in zebrafish. *Nat Methods* **6**, 733–735 (2009).

603 13. Le Bail, P. Y. *et al.* Optimization of somatic cell injection in the perspective of nuclear transfer in
604   goldfish. *BMC Dev Biol* **10**, 64 (2010).

605 14. Prukudom, S., Perez, G. I., Cibelli, J. B. & Siripattarapravat, K. Use of soluble sperm extract to
606   improve cloning efficiency in zebrafish. *Int J Dev Biol* **63**, 287–293 (2019).

607 15. Fatira, E. *et al.* A newly developed cloning technique in sturgeons; an important step towards
608   recovering endangered species. *Sci Rep* **9**, 10453 (2019).

609 16. Depince, A., Le Bail, P.-Y., Rouillon, C. & Labbe, C. Embryonic fate after somatic cell nuclear transfer
610   in non-enucleated goldfish oocytes is determined by first cleavages and DNA methylation
611   patterns. *Sci Rep* **11**, 3945 (2021).

612 17. Matoba, S. & Zhang, Y. Somatic Cell Nuclear Transfer Reprogramming: Mechanisms and
613   Applications. *Cell Stem Cell* **23**, 471–485 (2018).

614 18. Luo, D. *et al.* Identification of differentially expressed genes between cloned and zygote-
615   developing zebrafish (Danio rerio) embryos at the dome stage using suppression subtractive
616   hybridization. *Biol Reprod.* **80**, 674–684 (2009).

617 19. Depincé, A., Le Bail, P.-Y., Rouillon, C. & Labbé, C. Embryonic fate after somatic cell nuclear transfer
618   in non-enucleated goldfish oocytes is determined by first cleavages and DNA methylation
619   patterns. *Sci Rep* **11**, 3945 (2021).

620 20. Ganier, O. *et al.* Synergic reprogramming of mammalian cells by combined exposure to mitotic
621   Xenopus egg extracts and transcription factors. *Proc Natl Acad Sci USA* **108**, 17331–6 (2011).

622 21. Rathbone, A. J., Fisher, P. A., Lee, J. H., Craigon, J. & Campbell, K. H. S. Reprogramming of Ovine
623   Somatic Cells with Xenopus laevis Oocyte Extract Prior to SCNT Improves Live Birth Rate. *Cell
624   Reprogram* **12**, 609–616 (2010).

625 22. Liu, Y. *et al.* Improved Porcine Cloning Efficiency with Cells Cultured for Several Generations after
626   a Single Treatment with Xenopus Egg Extract. *Reprod Fert Develop* **23**, 130–131 (2011).

627 23. Yang, X. *et al.* Xenopus egg extract treatment reduced global DNA methylation of donor cells and
628   enhanced somatic cell nuclear transfer embryo development in pigs. *BioRes Open Access* **1**,
629   79–87 (2012).

630 24. Bru, T. *et al.* Rapid induction of pluripotency genes after exposure of human somatic cells to mouse
631   ES cell extracts. *Exp Cell Res* **314**, 2634–2642 (2008).

632 25. Miyamoto, K. *et al.* Reversible Membrane Permeabilization of Mammalian Cells Treated with
633   Digitonin and Its Use for Inducing Nuclear Reprogramming by Xenopus Egg Extracts. *Cloning
634   Stem Cells* **10**, 535–542 (2008).

635 26. Miyamoto, K. *et al.* Reprogramming events of mammalian somatic cells induced by Xenopus laevis
636   egg extracts. *Mol Reprod Dev* **74**, 1268–77 (2007).

637 27. Miyamoto, K. *et al.* Cell-Free Extracts from Mammalian Oocytes Partially Induce Nuclear
638   Reprogramming in Somatic Cells. *Biol Reprod* **80**, 935–943 (2009).

639 28. Hansis, C., Barreto, G., Maltry, N. & Niehrs, C. Nuclear reprogramming by Xenopus egg extract of
640   human somatic cells requires BRG1. *Curr Biol* **14**, 1475–1480 (2004).

18

641 29. Liu, Y. *et al.* Cell Colony Formation Induced by Xenopus Egg Extract as a Marker for Improvement
642   of Cloned Blastocyst Formation in the Pig. *Cell Reprogram* **13**, 521–526 (2011).

643 30. Zhu, X. Q. *et al.* Transient in vitro epigenetic reprogramming of skin fibroblasts into multipotent
644   cells. *Biomaterials* **31**, 2779–2787 (2010).

645 31. Chenais, N. *et al.* Nuclear import of Xenopus egg extract components into cultured cells for
646   reprogramming purposes: a case study on goldfish fin cells. *Sci Rep* **9**, 2861 (2019).

647 32. Rouillon, C., Depince, A., Chenais, N., Le Bail, P. Y. & Labbe, C. Somatic cell nuclear transfer in non-
648   enucleated goldfish oocytes: understanding DNA fate during oocyte activation and first cellular
649   division. *Sci Rep* **9**, 12462 (2019).

650 33. Marandel, L., Labbe, C., Bobe, J. & Le Bail, P.-Y. nanog 5'-upstream sequence, DNA methylation,
651   and expression in gametes and early embryo reveal striking differences between teleosts and
652   mammals. *Gene* **492**, 130–137 (2012).

653 34. Marandel, L. *et al.* Do not put all teleosts in one net: Focus on the sox2 and pou2 genes. *Comp
654   Biochem Physiol, Part B: Biochem Mol Biol* **164**, 69–79 (2013).

655 35. Alvarez, M. C., Béjar, J., Chen, S. & Hong, Y. Fish ES cells and applications to biotechnology. *Mar
656   Biotechnol (NY)* **9**, 117–127 (2007).

657 36. Yi, M., Hong, N. & Hong, Y. Derivation and characterization of haploid embryonic stem cell cultures
658   in medaka fish. *Nat Protoc* **5**, 1418–1430 (2010).

659 37. Vigneron, S. *et al.* Characterization of the Mechanisms Controlling Greatwall Activity. *Mol Cell Biol*
660   **31**, 2262–2275 (2011).

661 38. Castro, A. & Lorca, T. Greatwall kinase at a glance. *J Cell Sci.* **131**, jcs222364 (2018).

662 39. Liao, Y., Wang, J., Jaehnig, E. J., Shi, Z. & Zhang, B. WebGestalt 2019: gene set analysis toolkit with
663   revamped UIs and APIs. *Nucleic Acids Res* **47**, W199–W205 (2019).

664 40. Zhang, Y. E. Non-Smad pathways in TGF-beta signaling. *Cell Res* **19**, 128–139 (2009).

665 41. Shi, Y. & Massagué, J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell*
666   **113**, 685–700 (2003).

667 42. Ten Dijke, P., Goumans, M.-J., Itoh, F. & Itoh, S. Regulation of cell proliferation by Smad proteins.
668   *J Cell Physiol* **191**, 1–16 (2002).

669 43. Gordeeva, O. TGFβ Family Signaling Pathways in Pluripotent and Teratocarcinoma Stem Cells' Fate
670   Decisions: Balancing Between Self-Renewal, Differentiation, and Cancer. *Cells* **8**, (2019).

671 44. Onichtchouk, D. *et al.* Silencing of TGF-beta signalling by the pseudoreceptor BAMBI. *Nature* **401**,
672   480–485 (1999).

673 45. Kavsak, P. *et al.* Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta
674   receptor for degradation. *Mol Cell* **6**, 1365–1375 (2000).

675 46. Ichida, J. K. *et al.* A small-molecule inhibitor of tgf-Beta signaling replaces sox2 in reprogramming
676   by inducing nanog. *Cell Stem Cell* **5**, 491–503 (2009).

677 47. Li, R. *et al.* A mesenchymal-to-epithelial transition initiates and is required for the nuclear
678   reprogramming of mouse fibroblasts. *Cell Stem Cell* **7**, 51–63 (2010).

679 48. Lin, T. *et al.* A chemical platform for improved induction of human iPSCs. *Nat Methods* **6**, 805–808
680   (2009).

681 49. Maherali, N. & Hochedlinger, K. Tgfβ Signal Inhibition Cooperates in the Induction of iPSCs and
682   Replaces Sox2 and cMyc. *Curr Biol* **19**, 1718–1723 (2009).

683   50. Samavarchi-Tehrani, P. *et al.* Functional genomics reveals a BMP-driven mesenchymal-to-epithelial
684   transition in the initiation of somatic cell reprogramming. *Cell Stem Cell* **7**, 64–77 (2010).

685   51. Ke, Q. *et al.* Connexin 43 is involved in the generation of human-induced pluripotent stem cells.
686   *Hum Mol Genet* **22**, 2221–2233 (2013).

687   52. Lamouille, S., Xu, J. & Derynck, R. Molecular mechanisms of epithelial-mesenchymal transition.
688   *Nat Rev Mol Cell Biol* **15**, 178–96 (2014).

689   53. Grainger, S. & Willert, K. Mechanisms of Wnt signaling and control. *Wiley Interdiscip Rev Syst Biol*
690   *Med* e1422 (2018) doi:10.1002/wsbm.1422.

691   54. Sharma, G., Sharma, A. R., Seo, E.-M. & Nam, J.-S. Genetic polymorphism in extracellular regulators
692   of Wnt signaling pathway. *Biomed Res Int* **2015**, 847529 (2015).

693   55. Flowers, G. P., Topczewska, J. M. & Topczewski, J. A zebrafish Notum homolog specifically blocks
694   the Wnt/β-catenin signaling pathway. *Development* **139**, 2416–2425 (2012).

695   56. Mao, B. *et al.* Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling.
696   *Nature* **417**, 664–667 (2002).

697   57. Johansson, S., Svineng, G., Wennerberg, K., Keller, A. & Lohikangas, L. Fibronectin-integrin
698   interactions. *Front Biosci: J Virt Lib* **2**, d126-46 (1997).

699   58. Gradl, D., Kuhl, M. & Wedlich, D. The Wnt/Wg signal transducer beta-catenin controls fibronectin
700   expression. *Mol. Cell. Biol.* **19**, 5576–5587 (1999).

701   59. David, L. & Polo, J. M. Phases of reprogramming. *Stem Cell Research* **12**, 754–761 (2014).

702   60. Marandel, L., Labbe, C., Bobe, J. & Le Bail, P.-Y. Evolutionary history of c-myc in teleosts and
703   characterization of the duplicated c-myca genes in goldfish embryos. *Mol Reprod Dev* **79**, 85–
704   96 (2012).

705   61. Cheng, C., Geng, F., Cheng, X. & Guo, D. Lipid metabolism reprogramming and its potential targets
706   in cancer. *Cancer Commun (Lond)* **38**, 27 (2018).

707   62. Stadtfeld, M. & Hochedlinger, K. Induced pluripotency: history, mechanisms, and applications.
708   *Genes Dev* **24**, 2239–2263 (2010).

709   63. Jullien, J., Pasque, V., Halley-Stott, R. P., Miyamoto, K. & Gurdon, J. B. Mechanisms of nuclear
710   reprogramming by eggs and oocytes: a deterministic process? *Nat Rev Mol Cell Biol* **12**, 453–
711   459 (2011).

712   64. Farzaneh, M. *et al.* Suppression of TGF-β and ERK Signaling Pathways as a New Strategy to Provide
713   Rodent and Non-Rodent Pluripotent Stem Cells. *Curr Stem Cell Res Ther* **14**, 466–473 (2019).

714   65. Huh, S. *et al.* Suppression of the ERK-SRF axis facilitates somatic cell reprogramming. *Exp Mol Med*
715   **50**, e448 (2018).

716   66. Vidal, S. E., Amlani, B., Chen, T., Tsirigos, A. & Stadtfeld, M. Combinatorial modulation of signaling
717   pathways reveals cell-type-specific requirements for highly efficient and synchronous iPSC
718   reprogramming. *Stem Cell Rep* **3**, 574–584 (2014).

719   67. Jiao, J. *et al.* Promoting reprogramming by FGF2 reveals that the extracellular matrix is a barrier
720   for reprogramming fibroblasts to pluripotency. *Stem Cells* **31**, 729–740 (2013).

721   68. Aulicino, F., Theka, I., Ombrato, L., Lluis, F. & Cosma, M. P. Temporal Perturbation of the Wnt
722   Signaling Pathway in the Control of Cell Reprogramming Is Modulated by TCF1. *Stem Cell Rep*
723   **2**, 707–720 (2014).

724   69. Hong, H. *et al.* Suppression of induced pluripotent stem cell generation by the p53-p21 pathway.
725   *Nature* **460**, 1132–1135 (2009).

726 70. Esteban, M. A. & Pei, D. Vitamin C improves the quality of somatic cell reprogramming. *Nat Genet*
727   **44**, 366–367 (2012).

728 71. Chenais, N., Lareyre, J. J., Le Bail, P. Y. & Labbe, C. Stabilization of gene expression and cell
729   morphology 0 after explant recycling during fin explant culture in goldfish. *Exp Cell Res* **335**,
730   23–38 (2015).

731 72. Buganim, Y., Faddah, D. A. & Jaenisch, R. Mechanisms and models of somatic cell reprogramming.
732   *Nat Rev Genet* **14**, 427–439 (2013).

733 73. Wang, L. *et al.* Fatty acid synthesis is critical for stem cell pluripotency via promoting mitochondrial
734   fission. *EMBO J* **36**, 1330–1347 (2017).

735 74. Wu, Y. *et al.* Phospholipid remodeling is critical for stem cell pluripotency by facilitating
736   mesenchymal-to-epithelial transition. *Sci Adv* **5**, eaax7525 (2019).

737 75. Pei, Y. *et al.* Improvement in Mouse iPSC Induction by Rab32 Reveals the Importance of Lipid
738   Metabolism during Reprogramming. *Sci Rep* **5**, 16539 (2015).

739 76. Zhang, W. *et al.* Lipid Supplement in the Cultural Condition Facilitates the Porcine iPSC Derivation
740   through cAMP/PKA/CREB Signal Pathway. *Int J Mol Sci* **19**, (2018).

741 77. Fraher, D. *et al.* Zebrafish Embryonic Lipidomic Analysis Reveals that the Yolk Cell Is Metabolically
742   Active in Processing Lipid. *Cell Rep* **14**, 1317–1329 (2016).

743 78. Chenais, N., Depince, A., Le Bail, P.-Y. & Labbe, C. Fin cell cryopreservation and fish reconstruction
744   by nuclear transfer stand as promising technologies for preservation of finfish genetic
745   resources. *Aquacult Int* **22**, 63–76 (2014).

746 79. Edgar, R., Domrachev, M. & Lash, A. E. Gene Expression Omnibus: NCBI gene expression and
747   hybridization array data repository. *Nucleic Acids Res* **30**, 207–210 (2002).

748

749

750 **ACKNOWLEDGEMENTS**

757

758 **AUTHOR CONTRIBUTIONS STATEMENT**

759 NC designed the study, developed the experiments, analyzed the data, co-wrote the manuscript and

760 prepared the figures. ALC designed goldfish DNA microarray, conducted the microarray experiment

761 and provided preliminary statistical analysis of the microarray data. BG provided the indispensable

762 *Xenopus* eggs and contributed to the discussion. JJL provided expertise and help for the gene ontology

763 analysis and discussion. CL conceived the study and co-wrote the manuscript. All authors read and

764 approved the manuscript.

765

COMPETING INTERESTS

767 The authors declare no competing interests

768

DATA AVAILABILITY STATEMENT

770 Microarray data were published at the NCBI's Gene Expression Omnibus ([79] and are accessible through

771 GEO series accession number GSE205854. Most other data obtained in this work were provided in the

772 supplementary file. Any missing data or supplementary information should be asked to the

773 corresponding authors who will answer the requests in a timely manner.

774

FIGURE LEGENDS

776 Figure 1. Characteristics of the cultured fin cells after Xenopus egg extract treatment. Treated cells

777 were cultured in L15 (left) or ESM4 (right) media at 25 °C. Control cells (central) were neither

778 permeabilized nor exposed to egg extract and they were cultured in the same conditions as the treated

779 ones. Cell behavior and morphology were assessed by phase contrast microscopy after 2 (d2) and 7

780 (d7) days of culture. White arrows in d2 pictures: examples of cells with a round shape morphology,

781 contrasting with the elongated control cells. Inset : typical morphology of confluent control cells after

782 7 days in L15 medium. At d7, treated cells quality was higher in ESM4 (d7, right) than in L15 (d7, left)

783 medium. Pictures are representative of three experiments with different cells and egg extract batches.

784 Scale bar = 10 μm.

785

786 Figure 2. Hierarchical clustering analysis by unsupervised approach using 52,362 goldfish genes.

787 Control: cultured control cells (C1-5); Treated: cultured treated cells (T1-7). Each row represents a

788 single gene. Differentially expressed genes (Fold Change > 2; False Discovery Rate (FDR) < 0.05)

789 between treated and control cells are shown on the heatmap (2,286 genes). Two clusters were

790 identified. Cluster-I (872 genes) and cluster-II (1414 genes) contain the genes that were respectively

791 up- and down-regulated in treated cells compared to control cells.

792

793 Figure 3. Gene Ontology (GO) flow diagram of the terms related to cell surface receptor signaling

794 pathway (A) and KEGG pathways (B). The analysis was performed on the cluster of upregulated genes

795 in treated cells (fold change >2) using WebGestalt web tool. The set of genes spotted on the microarray

796 was used as the reference gene list. Black and dotted lines (in A) represent respectively direct and

797 indirect connections between GO terms. Drexxxxx (in B) gives Danio rerio prefix of the KEGG identifier.

798 For each GO term and KEGG pathway, p values (P) below 0.05 and false discovery rate (FDR) below

22

799   0.05 are indicated. Both A and B highlight the disturbance of the TGFβ and Wnt signaling pathways in

800   response to egg extract treatment.

801   Figure 4. Gene Ontology (GO) flow diagram of the terms related to lipid metabolic process (A) and

802   KEGG pathways (B). The analysis was performed on the cluster of genes downregulated in treated cells

803   (fold change >2) using WebGestalt web tool. The set of genes spotted on the microarray was used as

804   the reference gene list. A: The black and the dotted lines represent respectively direct and indirect

805   connections between GO terms. B: Dre, Danio rerio prefix of the KEGG identifier. For each GO term

806   and KEGG pathway, p values (P) below 0.05 and false discovery rate (FDR) below 0.05 are indicated.

807   Both A and B highlight the disturbance of lipid metabolism after egg extract treatment, and specifically

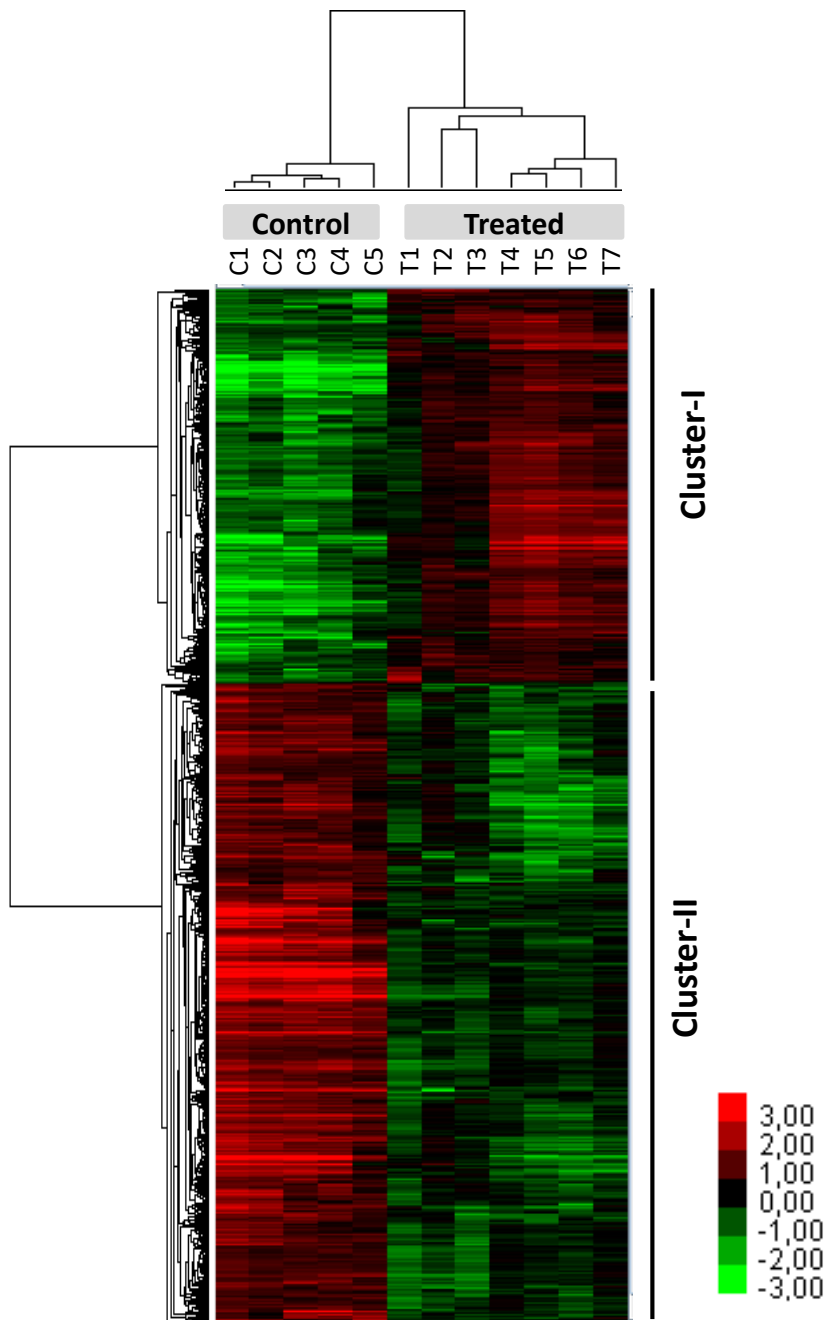808   cholesterol and fatty acid biosynthesis.

809

810

FIGURES AND TABLES
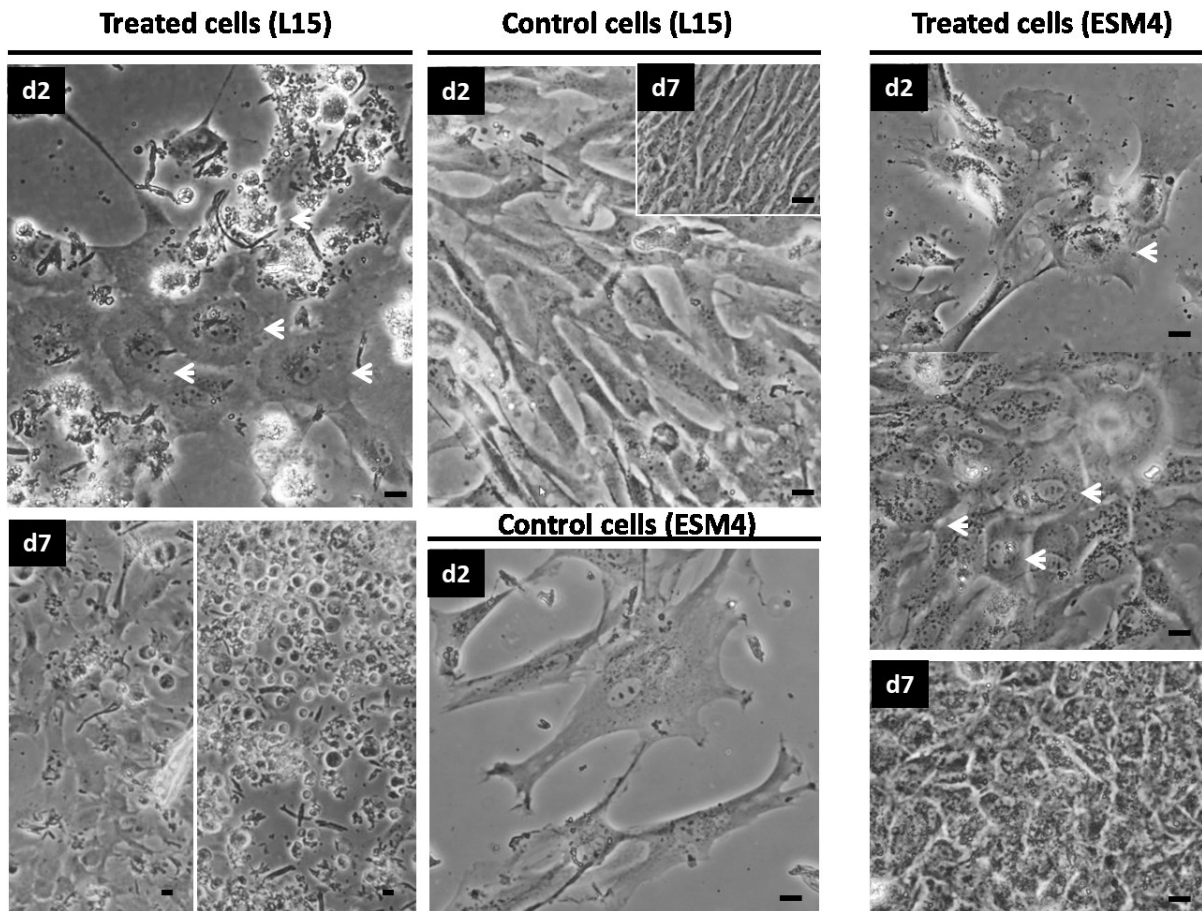
Figure 1. Characteristics of the cultured fin cells after Xenopus egg extract treatment. Treated cells were cultured in L15 (left) or ESM4 (right) media at 25 °C. Control cells (central) were neither permeabilized nor exposed to egg extract and they were cultured in the same conditions as the treated ones. Cell behavior and morphology were assessed by phase contrast microscopy after 2 (d2) and 7 (d7) days of culture. White arrows in d2 pictures: examples of cells with a round shape morphology, contrasting with the elongated control cells. Inset d7: typical morphology of confluent control cells after 7 days in L15 medium. At d7, treated cells quality was higher in ESM4 (d7, right) than in L15 (d7, left) medium. Pictures are representative of three experiments with different cells and egg extract batches. Scale bar = 10 μm.

24

824

Figure 2. Hierarchical clustering analysis by unsupervised approach using 52,362 goldfish genes. Control: cultured control cells (C1-5); Treated: cultured trea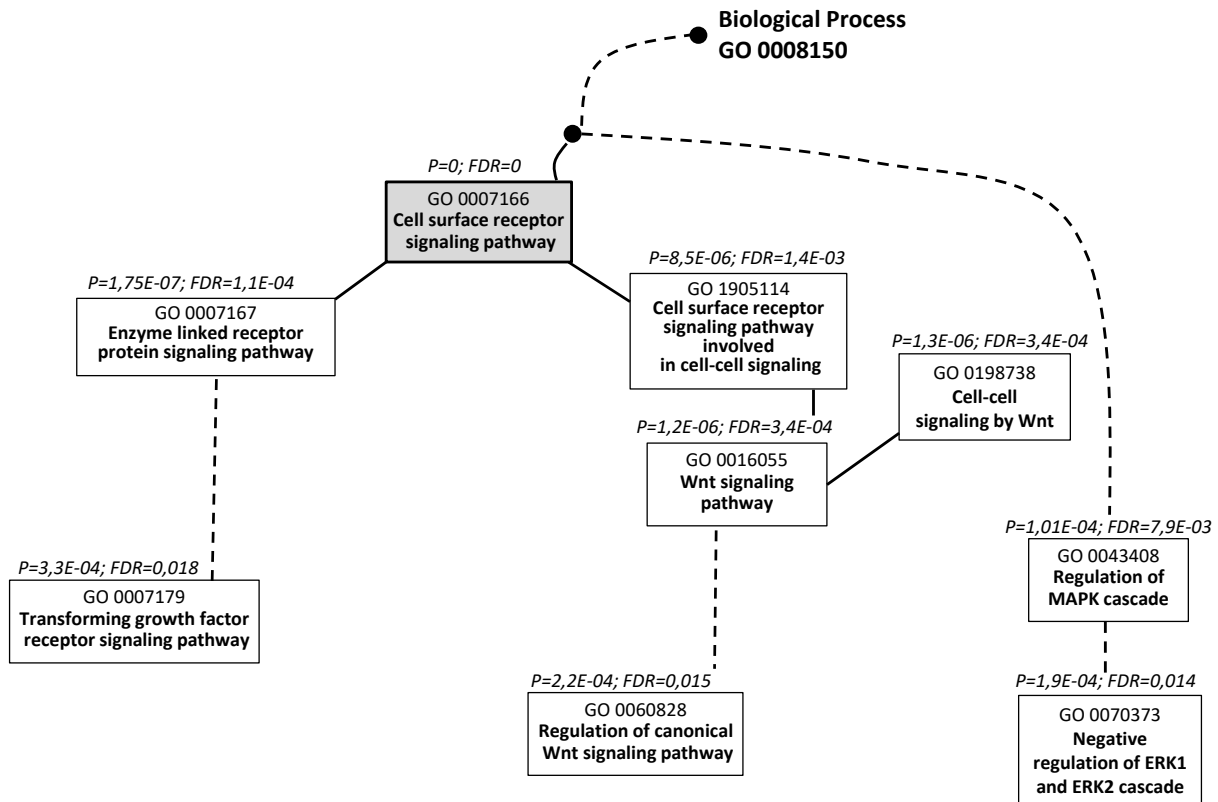ted cells (T1-7). Each row represents a single gene. Differentially expressed genes (Fold Change > 2; False Discovery Rate (FDR) < 0.05) between treated and control cells are shown on the heatmap (2,286 genes). Two clusters were identified. Cluster-I (872 genes) and cluster-II (1414 genes) contain the genes that were respectively up- and down-regulated in treated cells compared to control cells.

A

B

KEGG pathways		Gene Number	Enrichment ratio	P value	FDR
Dre04350	TGF-beta signaling pathway	14	4,83	8,52E-07	1,37E-04
Dre04310	Wnt signaling pathway	14	2,97	2,34E-04	1,88E-02

Figure 3. Gene Ontology (GO) flow diagram of the terms related to cell surface receptor signaling pathway (A) and KEGG pathways (B). The analysis was performed on the cluster of upregulated genes in treated cells (fold change >2) using WebGestalt web tool. The set of genes spotted on the microarray was used as the reference gene list. Black and dotted lines (in A) represent respectively direct and indirect connections between GO terms. Drexxxxx (in B) gives Danio rerio prefix of the KEGG identifier. For each GO term and KEGG pathway, p values (P) below 0.05 and false discovery rate (FDR) below 0.05 are indicated. Both A and B highlight the disturbance of the TGFβ and Wnt signaling pathways in response to egg extract treatment.

		Gene Symbol Danio rerio	Fold Change (treated *vs.* control)	Location	Description
TGFß Effectors	TGF-ß ligands	tgfb2	6,7	extra-cellular	transforming growth factor, beta 2 [Source:ZFIN;Acc:ZDB-GENE-030723-3]
		tgfb5	2,7	extra-cellular	transforming growth factor, beta 5 [Source:ZFIN;Acc:ZDB-GENE-130425-3]
	TGF-ß receptors	tgfbr1a	2,8 / 3,5	membrane	transforming growth factor, beta receptor 1 a [Source:ZFIN;Acc:ZDB-GENE-051120-75]
		tgfbr1b	2,2 / 2,2 / 2	membrane	transforming growth factor, beta receptor 1 b [Source:ZFIN;Acc:ZDB-GENE-091027-1]
	BMP ligands	bmp2a	3,2 / 2,8 / 2,8 / 2,8	extra-cellular	bone morphogenetic protein 2a [Source:ZFIN;Acc:ZDB-GENE-980526-388]
		bmp6	2,8 / 3,1	extra-cellular	bone morphogenetic protein 6 [Source:ZFIN;Acc:ZDB-GENE-050306-42]
		bmp7a	5,4	extra-cellular	bone morphogenetic protein 7a [Source:ZFIN;Acc:ZDB-GENE-000208-25]
	BMP receptor	bmpr1a	4,4	membrane	bone morphogenetic protein receptor, type IBa [Source:NCBI gene;Acc:30742]
TGFß Inhibitors	**upstream of signaling pathway**	bambia	3,2 / 2,7	membrane	BMP and activin membrane-bound inhibitor (Xenopus laevis) homolog a [Source:ZFIN;Acc:ZDB-GENE-010416-1]
		bambib	3,1	membrane	BMP and activin membrane-bound inhibitor homolog (Xenopus laevis) b [Source:ZFIN;Acc:ZDB-GENE-040704-30]
		lft2	4,9	extra-cellular	lefty2 [Source:ZFIN;Acc:ZDB-GENE-990630-11]
		grem2a	4,2 / 4,1	extra-cellular	gremlin 2, DAN family BMP antagonist a [Source:ZFIN;Acc:ZDB-GENE-131127-498]
		grem2b	3,5	extra-cellular	gremlin 2, DAN family BMP antagonist b [Source:ZFIN;Acc:ZDB-GENE-030911-9]
		nog1	2,6 / 2,6 / 2,4	extra-cellular	noggin 1 [Source:NCBI gene;Acc:30174]
		nog2	2,3	extra-cellular	noggin 2 [Source:NCBI gene;Acc:30185]
	canonical signaling pathway	smad6a	4, 1 / 2,8	intra-cellular	SMAD family member 6a [Source:ZFIN;Acc:ZDB-GENE-011015-1]
		smad6b	2,3 / 2,2	intra-cellular	SMAD family member 6b [Source:ZFIN;Acc:ZDB-GENE-050419-198]
		smad7	2,9 / 2,9 / 2,8	intra-cellular	SMAD family member 7 [Source:ZFIN;Acc:ZDB-GENE-030128-3]
		smad9	2,9 / 2,4	intra-cellular	SMAD family member 9 [Source:ZFIN;Acc:ZDB-GENE-031014-1]
		smurf2	2,1	intra-cellular	SMAD specific E3 ubiquitin protein ligase 2 [Source:NCBI gene;Acc:563633]
	non canonical signaling pathway	spry1	3,2	intra-cellular	sprouty homolog 1, antagonist of FGF signaling (Drosophila) [Source:ZFIN;Acc:ZDB-GENE-081215-2]
		spry4	4,5 / 3,4	intra-cellular	sprouty homolog 4 (Drosophila) [Source:ZFIN;Acc:ZDB-GENE-010803-2]
		dusp6	5,8 / 5,6 / 4,2	intra-cellular	dual specificity phosphatase 6 [Source:ZFIN;Acc:ZDB-GENE-030613-1]

845

846 Table 1. List of the TGFβ signaling actors that were upregulated in treated cells. For each Danio rerio

847 gene symbol, Fold Change values are given for all the corresponding isoforms found in goldfish. Despite

848 upregulation of some TGFβ effectors, upregulation of many inhibitors acting upstream and

849 downstream of the signaling pathway are signing TGFβ inhibition in treated cells. Both canonical and

850 non-canonical signaling pathways were affected.

		Gene Symbol *Danio rerio*	Fold Change (treated *vs.* control)	Direction of Regulation	Description
	Transcription Factor	zeb1	2,2 / 3,3	down	zinc finger E-box binding homeobox 1b [Source:ZFIN;Acc:ZDB-GENE-010621-1]
TGF-ß target genes	mesenchymal marker genes	fn1	**43,7**	down	fibronectin 1b [Source:ZFIN;Acc:ZDB-GENE-030131-6545]
		col5a1	2,1 / 3,9 / 4 / 4,1 / 3,4 / 2,3	down	procollagen, type V, alpha 1 [Source:ZFIN;Acc:ZDB-GENE-041105-6]
		col6a1	4,2 / 3,2	down	collagen, type VI, alpha 1 [Source:ZFIN;Acc:ZDB-GENE-070501-6]
		col6a2	3,1 / 3,2 / 3,1	down	collagen, type VI, alpha 2 [Source:ZFIN;Acc:ZDB-GENE-070501-7]
		col6a3	3,9 / 3,6 / 3,4 / 3,3 / 2,9	down	collagen, type VI, alpha 3 [Source:ZFIN;Acc:ZDB-GENE-070501-8]
		col8a2	2,7 / 2,9	down	collagen, type VIII, alpha 2 [Source:ZFIN;Acc:ZDB-GENE-060503-488]
		col12a1b	2,8	down	collagen, type XII, alpha 1b [Source:ZFIN;Acc:ZDB-GENE-120215-116]
		col16a1	4,6 / 6	down	collagen, type XVI, alpha 1 [Source:ZFIN;Acc:ZDB-GENE-060503-351]
		col21a1	9,1 / 6,2	down	collagen, type XXI, alpha 1 [Source:ZFIN;Acc:ZDB-GENE-110607-3]
		col27a1b	4,4 / 3,9	down	collagen, type XXVII, alpha 1b [Source:NCBI gene;Acc:560145]
		col28a1b	3,4	down	collagen, type XXVIII, alpha 1b [Source:ZFIN;Acc:ZDB-GENE-160503-1]
		pcdh2aa1	2,1	down	protocadherin 2 alpha a 1 [Source:ZFIN;Acc:ZDB-GENE-041118-13]
		pcdh2ab9	2,03/ 2,1/ 2,1 / 2,3 / 2,4 / 3,1	down	protocadherin 2 alpha b 9 [Source:ZFIN;Acc:ZDB-GENE-041118-8]
		mmp9	12	down	matrix metallopeptidase 9 [Source:ZFIN;Acc:ZDB-GENE-040426-2132]
	epithelial marker genes	pcdh1b	2,3 / 3,3	up	protocadherin 1b [Source:ZFIN;Acc:ZDB-GENE-091015-2]
		pcdh12	4,8	up	protocadherin 12 [Source:ZFIN;Acc:ZDB-GENE-140106-126]
		cdh24b	8,8	up	cadherin 24, type 2b [Source:ZFIN;Acc:ZDB-GENE-081104-50]
		krt15	5,8	up	keratin 15 [Source:ZFIN;Acc:ZDB-GENE-040426-2931]
		krt18a1	2,4	up	keratin 18a, tandem duplicate 1 [Source:NCBI gene;Acc:352912]
		pkp3b	4,1	up	plakophilin 3b [Source:ZFIN;Acc:ZDB-GENE-130530-870]
		cldn5a	3,4	up	claudin 5a [Source:ZFIN;Acc:ZDB-GENE-040426-2442]
		tjp1a	2	up	tight junction protein 1a [Source:ZFIN;Acc:ZDB-GENE-031001-2]
		cx43	2,1	up	connexin 43 [Source:NCBI gene;Acc:30236]

851

852   Table 2. List of the TGFβ target genes related to mesenchymal-epithelial transition (MET) that were

853   differentially expressed between treated and control cells. For each Danio rerio symbol gene, Fold

854   Change values (up and down) are given for all the corresponding isoforms found in goldfish. Treated

855   cells were characterized by the downregulation of several mesenchymal markers, especially fn1 and

856   collagens, and by the upregulation of several epithelial markers, suggesting a MET.

|		Gene Symbol *Danio rerio*	Fold Change (treated *vs.* control)	Direction of Regulation	Location	Description
Wnt effectors	Ligands	wnt5b	2,1	up	extra-cellular	wingless-type MMTV integration site family, member 5b [Source:NCBI gene;Acc:30105]
		wnt7bb	3,6 / 3,6 / 3,9 / 4,4	up	extra-cellular	wingless-type MMTV integration site family, member 7Bb [Source:ZFIN;Acc:ZDB-GENE-081006-1]
		wnt9a	3,9 / 4	up	extra-cellular	wingless-type MMTV integration site family, member 9A [Source:ZFIN;Acc:ZDB-GENE-060825-97]
	Receptors	fzd8a	2,5	up	membrane	frizzled class receptor 8a [Source:ZFIN;Acc:ZDB-GENE-000328-3]
		fzd10	18,1 / 25,9	up	membrane	frizzled class receptor 10 [Source:ZFIN;Acc:ZDB-GENE-990415-220]
	Wnt agonists	rspo2	3,2 / 3,6	up	extra-cellular	R-spondin 2 [Source:ZFIN;Acc:ZDB-GENE-060503-667]
		rspo3	5,4 / 7,5	up	extra-cellular	R-spondin 3 [Source:NCBI gene;Acc:100007702]
Wnt Inhibitors		bambia	3,2 / 2,7	up	membrane	BMP and activin membrane-bound inhibitor (Xenopus laevis) homolog a [Source:ZFIN;Acc:ZDB-GENE-010416-1]
		bambib	3,1	up	membrane	BMP and activin membrane-bound inhibitor homolog (Xenopus laevis) b [Source:ZFIN;Acc:ZDB-GENE-040704-30]
		notum1a	4 / 10,4	up	extra-cellular	notum, palmitoleoyl-protein carboxylesterase a [Source:NCBI gene;Acc:570510]
		frzb	7,15	up	extra-cellular	frizzled related protein [Source:ZFIN;Acc:ZDB-GENE-990715-1]
		sfrp1a	3,75	down	extra-cellular	secreted frizzled-related protein 1a [Source:ZFIN;Acc:ZDB-GENE-040310-5]
		sfrp1b	7,5	down	extra-cellular	secreted frizzled-related protein 2 [Source:ZFIN;Acc:ZDB-GENE-061013-293]
		sost	6 / 7 / 8,7	up	extra-cellular	sclerostin [Source:NCBI gene;Acc:100000500]
		dkk1a	5,5 / 9,5	down	extra-cellular	dickkopf WNT signaling pathway inhibitor 1a [Source:ZFIN;Acc:ZDB-GENE-090313-406]
		dkk1b	3,1	up	extra-cellular	dickkopf WNT signaling pathway inhibitor 1b [Source:ZFIN;Acc:ZDB-GENE-990708-5]
		kremen1	2,1	up	membrane	kringle containing transmembrane protein 1 [Source:NCBI gene;Acc:100141352]
		gpc4	2,1	up	membrane	glypican 4 [Source:ZFIN;Acc:ZDB-GENE-011119-1]
Others Wnt actors	Transcription factors	tcf7	3,9	up	nucleus	transcription factor 7 [Source:ZFIN;Acc:ZDB-GENE-050222-4]
		tcf7l1a	2,3 / 3,2	down	nucleus	transcription factor 7 like 1a [Source:NCBI gene;Acc:30523]
		tcf7l1b	2	down	nucleus	transcription factor 7 like 1b [Source:NCBI gene;Acc:30556]
	Target genes	pak1	2,2	down	-	p21 protein (Cdc42/Rac)-activated kinase 1 [Source:ZFIN;Acc:ZDB-GENE-030826-29]
		fn1	43,7	down	-	fibronectin 1b [Source:ZFIN;Acc:ZDB-GENE-030131-6545]

857

858

859 Table 3. List of the Wnt/β-catenin signaling actors that were differentially expressed between treated

860 and control cells. For each Danio rerio symbol gene, Fold Change values (up and down) are given for

861 all the corresponding isoforms found in goldfish. The overall disturbance of the Wnt/ β-catenin

862 signaling pathway in response to egg extract treatment, indicated by up/down regulation of effectors,

863 inhibitors and transcription factors, tilts towards Wnt signaling inhibition.

	Gene Symbol *Danio rerio*	Fold Change (Treated *vs.* control)	Description
Acetyl-CoA synthesis	slc25a1b	6,6 / 11,7 / 14,2	slc25a1 solute carrier family 25 member 1b [Source:NCBI gene;Acc:795332]
	aclya	4,4 / 4,5	ATP citrate lyase a [Source:ZFIN;Acc:ZDB-GENE-031113-1]
	acss2l	6,63 / 32,6	acyl-CoA synthetase short chain family member 2 like [Source:ZFIN;Acc:ZDB-GENE-130530-723]
	acsl3a	59,8	acyl-CoA synthetase long chain family member 3a [Source:ZFIN;Acc:ZDB-GENE-050420-181]
	acat2	3 / 5	acetyl-CoA acetyltransferase 2 [Source:ZFIN;Acc:ZDB-GENE-990714-22]
Fatty acid biosynthesis	fasn	2,8 / 2,8 / 3,4	fatty acid synthase [Source:ZFIN;Acc:ZDB-GENE-030131-7802]
	scd	6,5 / 6,9 / 7,4	stearoyl-CoA desaturase (delta-9-desaturase) [Source:ZFIN;Acc:ZDB-GENE-031106-3]
	fads2	32,2 / 34,8 / 35 / 35,6	fatty acid desaturase 2 [Source:NCBI gene;Acc:140615]
	elov1a	2,2 / 2,4	ELOVL fatty acid elongase 1a [Source:ZFIN;Acc:ZDB-GENE-041010-66]
	elov2	3 / 22,3	ELOVL fatty acid elongase 2 [Source:ZFIN;Acc:ZDB-GENE-060421-5612]
	elov5	2,8 / 5,5	ELOVL fatty acid elongase 5 [Source:ZFIN;Acc:ZDB-GENE-040407-2]
	elov6	2,1 / 9,6	ELOVL fatty acid elongase 6 [Source:NCBI gene;Acc:317738]
Cholesterol biosynthesis	hmgcs1	13,2 / 12,4	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble) [Source:ZFIN;Acc:ZDB-GENE-040426-1042]
	hmgcra	5,1 /5,2	3-hydroxy-3-methylglutaryl-CoA reductase a [Source:ZFIN;Acc:ZDB-GENE-040401-2]
	msmo1	22,6	methylsterol monooxygenase 1 [Source:NCBI gene;Acc:406662]
	fdft1	6,3 / 6,7 / 6,7	farnesyl-diphosphate farnesyltransferase 1 [Source:ZFIN;Acc:ZDB-GENE-081104-242]
	cyp51	10,6 /10,9	cytochrome P450, family 51 [Source:NCBI gene;Acc:414331]
	dhcr7	8,4 / 8,8	7-dehydrocholesterol reductase [Source:NCBI gene;Acc:378446]
	dhcr24	8,1	24-dehydrocholesterol reductase [Source:ZFIN;Acc:ZDB-GENE-041212-73]
	cyp7a1	4,6 / 5,5	cytochrome P450, family 7, subfamily A, polypeptide 1 [Source:ZFIN;Acc:ZDB-GENE-040426-1296]
	mvda	4,4	mevalonate (diphospho) decarboxylase a [Source:NCBI gene;Acc:492781]
Transcription factors *	srebf1	3,3 / 4	sterol regulatory element binding transcription factor 1 [Source:ZFIN;Acc:ZDB-GENE-090812-3]
	Srebf2	2,6 / 2,8	sterol regulatory element binding transcription factor 2 [Source:NCBI gene;Acc:100037309]

864

865 Table 4. List of the genes associated with lipid biosynthesis that were downregulated in treated cells.

866 For each *Danio rerio* symbol gene, Fold change values are given for all the corresponding isoforms

867 found in goldfish. *: transcription factors involved in the regulation of fatty acid and cholesterol *de*

868 *novo* synthesis.

A
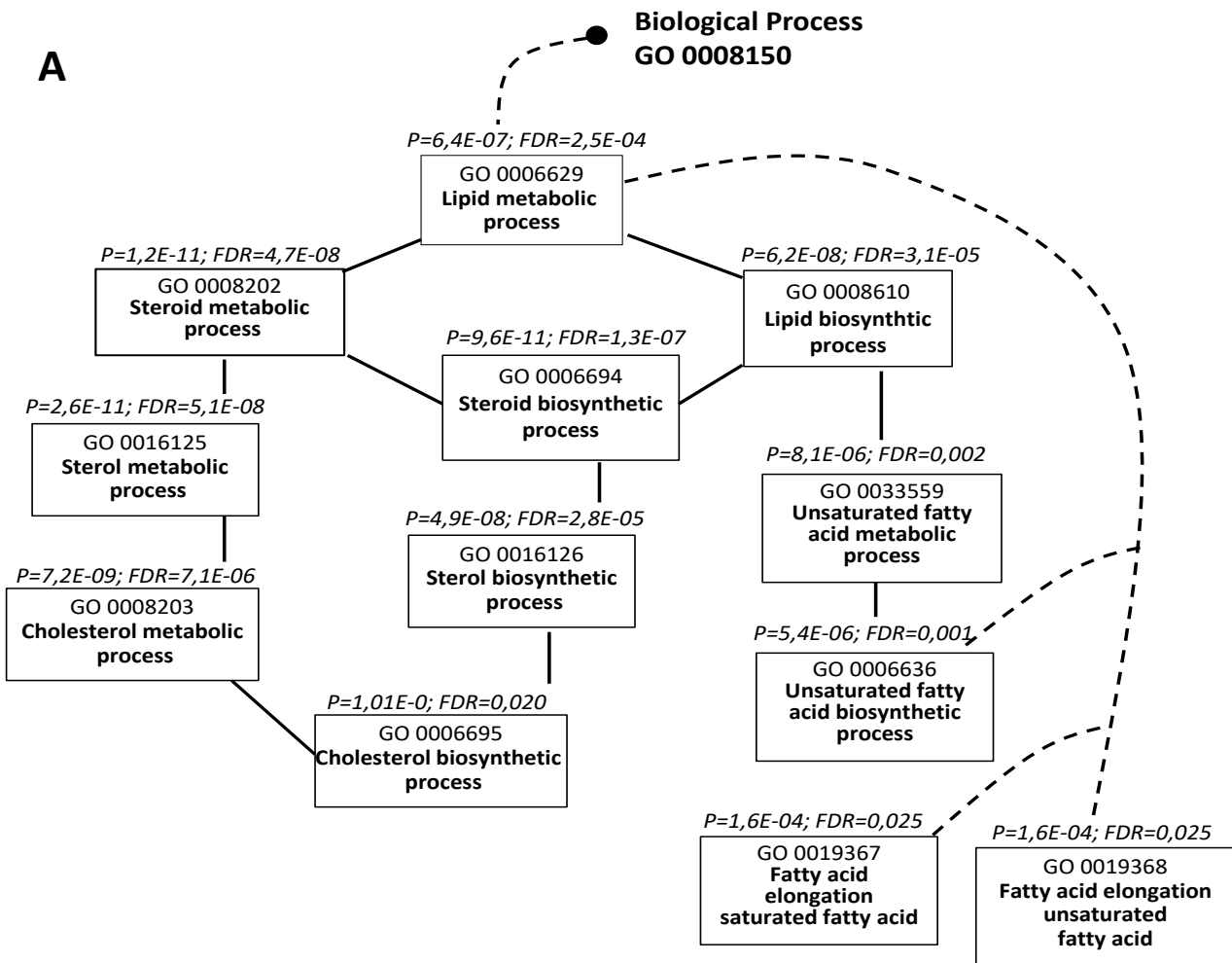
869

870 **B**

KEGG pathways		Gene Number	Enrichment ratio	P value	FDR
Dre00100	Steroid biosynthesis	12	14,15	1,34E-12	2,16E-10
Dre01040	Biosynthesis of unsaturated fatty acids	6	4,89	1,07E-03	2,46E-02
Dre01212	Fatty acid metabolism	9	3,67	6,15E-04	2,46E-02

871 Figure 4. Gene Ontology (GO) flow diagram of the terms related to lipid metabolic process (A) and

872 KEGG pathways (B). The analysis was performed on the cluster of genes downregulated in treated cells

873 (fold change >2) using WebGestalt web tool. The set of genes spotted on the microarray was used as

874 the reference gene list. A: The black and the dotted lines represent respectively direct and indirect

875 connections between GO terms. B: Dre, Danio rerio prefix of the KEGG identifier. For each GO term

876 and KEGG pathway, p values (P) below 0.05 and false discovery rate (FDR) below 0.05 are indicated.

877 Both A and B highlight the disturbance of lipid metabolism after egg extract treatment, and specifically

878 cholesterol and fatty acid biosynthesis.