Metabolism and Sex Differentiation in Animals from a Starvation Perspective

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Keywords

Metabolism · Pantothenate · Sex differentiation · Sex reversal · Starvation

Abstract

Animals determine their sex genetically (GSD: genetic sex determination) and/or environmentally (ESD: environmental sex determination). Medaka (*Oryzias latipes*) employ a XX/XY GSD system, however, they display female-to-male sex reversal in response to various environmental changes such as temperature, hypoxia, and green light. Interestingly, we found that 5 days of starvation during sex differentiation caused female-to-male sex reversal. In this situation, the metabolism of pantothenate and fatty acid synthesis plays an important role in sex reversal. Metabolism is associated with other biological factors such as germ cells, HPG axis, lipids, and epigenetics, and supplies substances and acts as signal transducers. In this review, we discuss the importance of metabolism during sex differentiation and how metabolism contributes to sex differentiation.

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Introduction

Starvation is an environmental ordeal any animal can face. This requires animals to trade-off survival and reproduction [Kalra and Parkash, 2014; Lynn et al., 2015]. Starvation and dietary restriction extend lifespan and suppress reproductive events such as reproductive cycle

and gametogenesis in nematoda, fly, duck, chicken, cattle, sheep, mouse, and human [Hosoda et al., 1955; Van der Spuy, 1985; Schillo, 1992; Drummond-Barbosa and Spradling, 2001; Elias et al., 2007; Angelo and Van Gilst, 2009; Song et al., 2009; Della Torre et al., 2011; Manfredi-Lozano et al., 2018]. The environment also affects sex in several species of fish and reptiles despite having a genetic sex determination (GSD) system (Table 1). This may imply that the animals have an alternative sex determination mechanism in response to environmental factors such as pH, photoperiod, hypoxia, temperature, and density. However, the molecular mechanism underlying environmental sex determination (ESD) is not fully understood. In fish, high temperature and background color induce sex reversal and change the level of cortisol (known as the stress hormone) [Hattori et al., 2009; Hayashi et al., 2010; Yamaguchi et al., 2010; Mankiewicz et al., 2013]. In reptiles, many species have temperature-dependent sex determinations (TSD) [Kohno et al., 2014], but the involvement of cortisol has not been reported so far (Table 1). Therefore, we are not sure whether the involvement of cortisol in sex differentiation is common in these species.

In reptiles with TSD, the temperature sensor TRPV4 and the histone demethylase KDM6B are involved [Yatsu et al., 2015; Ge et al., 2018; Weber et al., 2020]. In *Daphnia* plankton (*Daphnia pulex*), the elevation of pantothenate plays an important role in photoperiod-dependent male production [Toyota et al., 2016]. However, as briefly described above, our knowledge of the mechanism of ESD remains fragmentary.

Table 1. Environmental factors which affect sex determination or sex differentiation

Environmental factor	Species	GSD/ESD	Relating molecule	Reference
Temperature	Medaka (<i>Oryzias latipes</i>)	GSD	Cortisol	Hayashi et al. [2010]
	Pejerrey (<i>Odontesthes bonariensis</i>)	GSD, ESD	Cortisol	Hattori et al. [2009]
	Zebrafish (<i>Danio rerio</i>)	GSD, ESD	Unknown	Abozaid et al. [2011]
	Japanese flounder (<i>Paralichthys olivaceus</i>)	GSD, ESD	Cortisol	Yamaguchi et al. [2010]
	European sea bass (<i>Dicentrarchus labrax</i>)	GSD, ESD	Unknown	Saillant et al. [2002]
	Red-eared slider turtle (<i>Trachemys scripta</i>)	ESD	STAT3, KDM6B	Ge et al. [2018]; Weber et al. [2020]
	Tropical freshwater turtle (<i>Malayemys macrocephala</i>)	ESD	Unknown	Pewphong et al. [2020]
	Gekkonidae (<i>Gekko japonicus</i>)	ESD	Unknown	Tokunaga [1985]
	Eastern three-lined skink (<i>Bassiana duperreyi</i>)	GSD, ESD	Unknown	Radder et al. [2008]
	Australian bearded dragon (<i>Pogona vitticeps</i>)	GSD, ESD	Unknown	Holleley et al. [2015]
	American alligator (<i>Alligator mississippiensis</i>)	ESD	TRPV4	Yatsu et al. [2015]
Photoperiod	California grunion (<i>Leuresthes tenuis</i>)	GSD, ESD	Unknown	Brown et al. [2014]
	Daphnia (<i>Daphnia pulex</i>)	ESD	Pantothenate, methyl farnesoate, NMDAR	Toyota et al. [2015a, b, 2016]
	Daphnia (<i>Daphnia magna</i>)	ESD	NMDAR, methyl farnesoate	Toyota et al. [2021]
Green light	Medaka (<i>Oryzias latipes</i>)	GSD	Unknown	Hayasaka et al. [2019]
Hypoxia	Medaka (<i>Oryzias latipes</i>)	GSD	Unknown	Cheung et al. [2014]
	Zebrafish (<i>Danio rerio</i>)	GSD, ESD	Unknown	Shang et al. [2006]
pH	Cichlids (<i>Pelvicachromis pulcher</i> , <i>Pelvicachromis subocellatus</i> , <i>Pelvicachromis taeniatus</i> , <i>Apistogramma borellii</i> , <i>Apistogramma cacatuoides</i> , <i>Xiphophorus hellerii</i>)	ESD	Unknown	Rubin [1985]
Density	Zebrafish (<i>Danio rerio</i>)	GSD, ESD	Unknown	Ribas et al. [2017]
Density, food concentration	Daphnia (<i>Daphnia magna</i>)	ESD	Unknown	Olmstead and Leblanc [2001]
Background color	Southern flounder (<i>Paralichthys lethostigma</i>)	GSD, ESD	Cortisol	Mankiewicz et al. [2013]
Food condition	Bivalves (<i>Mytella charruana</i>)	–	Unknown	Stenyakina et al. [2010]
	Zebrafish (<i>Danio rerio</i>)	GSD, ESD	Unknown	Lawrence et al. [2008]

Interestingly, feeding conditions contribute to sex in zebrafish (*Danio rerio*) and bivalves (*Mytella charruana*) [Lawrence et al., 2008; Stenyakina et al., 2010]. Although the mechanism underlying sex regulation according to feeding conditions was unclear, these reports suggest that metabolism and metabolites may participate in sex regulation. It is recently reported that metabolism is involved in stem cell maintenance and differentiation [Folmes et al., 2012; Folmes and Terzic, 2015; Harvey et al., 2019]. This would be a good example of how metabolism is actively involved in biological phenomena as signals, more than just reactions and products. Therefore, knowledge from stem cell studies would give us an idea of how metabolism contributes to biological phenomena.

In this review, we first explain the general effects of starvation on metabolism. Next, we show how the metabolism makes an effect on stem cells as an example. We

then describe sex reversal mediated by starvation that we found using medaka, with an emphasis on the importance of metabolism in sex determination and sex differentiation induced by the environment.

Overview of the Effect of Starvation on Metabolism

When exposed to starvation, animals change their metabolism in order to survive. The most well-studied metabolic process that responds to starvation is the one that produces energy. Glucose, an important nutrient for energy (ATP) production, is depleted under starvation conditions [McCue, 2010]. To compensate for the low ATP state upon depletion of glucose, glycogen stored in the liver is first catabolized [Anyamaneratch et al., 2015]. Although amino acids are supplied by protein degradation and used in ATP

Table 2. Contribution of metabolites and metabolism to stem cells

Metabolites/metabolisms		Stem cells	Contribution	Reference
Sugars	FBP, PEP	MSC (human)	Self-renewal	Jeong et al. [2019]
Amino acids	Methionine	ESC, iPSC (human)	Self-renewal, survival, stemness	Shiraki et al. [2014]
	Glutamine	HSC (mouse)	Differentiation	Oburoglu et al. [2014]
	Threonine	ESC (mouse)	Self-renewal	Wang et al. [2009]
	L-Proline	ESC (mouse)	Stemness	Washington et al. [2010]; Casalino et al. [2011]
	Valine	HSC (human, mouse)	Self-renewal	Taya et al. [2016]
Lipids	Butyric acid	iPSC (human)	Reprogramming efficiency	Mali et al. [2010]
	Oleic acid	ESC (mouse)	Self-renewal, stemness	Wang et al. [2017]
	Palmitic acid, capric acid	ESC (human, mouse)	Differentiation	Yanes et al. [2010]
	Linoleic acid	ESC (mouse)	Self-renewal	Kim et al. [2009]
	DHA	NSC (mouse)	Differentiation	Kawakita et al. [2006]
	ARA	NSPC (mouse)	Differentiation	Sakayori et al. [2011]
	EPA	NSC (rat)	Differentiation	Katakura et al. [2013]
	PGE2	HSC (zebrafish, mouse)	Self-renewal	North et al. [2007]
	PGE2	ESC (mouse)	Self-renewal	Yun et al. [2009]
	15-Deoxy- Δ 12,14-Prostaglandin J2	ESC (mouse)	Self-renewal	Rajasingh and Bright [2006]
	Leukotriene D4	ESC (mouse)	Self-renewal	Kim et al. [2010]
	Neuroprotectin D1	ESC (mouse)	Differentiation	Yanes et al. [2010]
	Estradiol	HSC (mouse)	Self-renewal	Nakada et al. [2014]
Others	ROS	CMP (fly)	Self-renewal	Owusu-Ansah and Banerjee [2009]
	ROS	MSC (human)	Differentiation	Tormos et al. [2011]
	ROS	HSC (mouse)	Survival	Ito et al. [2006]; Tothova et al. [2007]
	Cyclic ADP ribose	ISC (mouse)	Self-renewal	Yilmaz et al. [2012]
	α -KG	ESC (mouse)	Differentiation	Tischler et al. [2019]
	α -KG	ESC (mouse)	Self-renewal, stemness	Carey et al. [2015]
	Acetate	ESC (human, mouse)	Self-renewal. Stemness	Moussaieff et al. [2015]
	Ascorbic acid	ESC (mouse)	Differentiation	Takahashi et al. [2003]
Glycolysis		Myoblast (mouse)	Self-renewal	Bracha et al. [2010]
		MSC (human)	Self-renewal	Jeong et al. [2019]
		iPSC (mouse)	Reprogramming efficiency (1)	Folmes et al. [2011]
		iPSC (human)	Reprogramming efficiency (1)	Zhu et al. [2010]
		SSC (mouse)	Self-renewal	Kanatsu-Shinohara et al. [2016]
OXPHOS		ESC (mouse)	Self-renewal	Mandal et al. [2010]
		iPSC (mouse)	Reprogramming efficiency (1)	Folmes et al. [2011]
		iPSC (human)	Reprogramming efficiency (1)	Zhu et al. [2010]
β -Oxidation		HSC (mouse)	Self-renewal	Ito et al. [2012]
Fatty acid synthesis		NSPC (mouse)	Self-renewal	Knobloch et al. [2013]
		ESC, iPSC (mouse)	Self-renewal, stemness	Wang et al. [2017]
Acetyl-CoA synthesis		Myoblast (mouse)	Self-renewal	Bracha et al. [2010]
Mevalonate pathway		HSC (human)	Self-renewal	Cimato et al. [2013]
		Myoblast (mouse)	Differentiation	Bracha et al. [2010]
Eicosanoid synthesis		ESC (mouse)	Differentiation	Yanes et al. [2010]

ARA, arachidonic acid; CMP, common myeloid progenitor; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ESC, embryonic stem cell; FBP, fructose-1,6-bisphosphate; HSC, hematopoietic stem cell; iPSC, induced pluripotent stem cell; ISC, intestinal stem cell; α -KG, α -ketoglutaric acid; MSC, mesenchymal stem cell; NSC, neural stem cell; NSPC, neural stem and progenitor cell; PEP, phosphoenolpyruvic acid; PGE2, prostaglandin E2; ROS, reactive oxygen species; SSC, spermatogonial stem cell.

production as substrates for gluconeogenesis and the TCA cycle under normal conditions, they rarely serve as an ATP source under starvation conditions [George and Owen, 1968; Black and Love, 1986; Hervant et al., 2001]. With exhausted glycogen, lipids become another energy source. Lipids (mainly neutral lipids) are catalyzed in mitochondria (β -oxidation), which causes an elevation in acetyl-CoA, a substrate for ATP production [Chandel, 2014]. Many animals, such as channel catfish, brook trout, Nile tilapia, clawed frog, Japanese quail, common eider, emperor penguin, chicken, and rat, catabolize lipids as a energy source under starvation conditions [McCue, 2010].

Metabolites Function as Stem Cell Regulators

Although metabolites are regarded as a by-product of life activity, recently there have been increasing reports that metabolites actively regulate biological phenomena. A well-known role is to provide building blocks. For instance, the pentose phosphate pathway (PPP) (the branching pathway of glycolysis) provides deoxyribose and ribose for nucleic acid synthesis, and glycolysis and the TCA cycle supply substrates for amino acid synthesis [Berg et al., 2002].

In addition, metabolites are known as regulators. One example is the regulation of stem cells [Folmes et al., 2012; Folmes and Terzic, 2015; Harvey et al., 2019]. Stem cells, such as tissue stem cells, embryonic stem (ES) cells, and induced pluripotent stem (iPS) cells, mainly depend on glycolysis for ATP synthesis as compared to differentiated cells, while their oxidative phosphorylation activity is low [Folmes et al., 2011]. This metabolic status is important for controlling the maintenance and acquisition of pluripotency and differentiation in stem cells [Panopoulos et al., 2012; Spyrou et al., 2019]. In the mechanisms, several metabolites, especially lipids, act as signaling factors to control stem cell self-renewal and differentiation (Table 2).

Metabolites also regulate the epigenetic status of stem cells. Analysis of micrococcal nuclease (MNase), DNase I, and the assay for transposase-accessible chromatin sequencing (ATAC-seq) revealed that ES cells show higher accessibility of chromatin and transcriptional factors in the genome than differentiated cells [Deng et al., 2013; Morozumi et al., 2016; Simon et al., 2017]. Supporting this, ES and iPS cells display a higher level of H3/H4 acetylation (euchromatin marker) and a lower level of H3K9me3 (heterochromatin marker) than is observed in differentiated cells [Sridharan et al., 2013]. This suggests that

stem cells are characterized as open chromatin in an epigenetic manner [Schlesinger and Meshorer, 2019].

An epigenomic difference is also involved in the features of stem cells [Lunyak and Rosenfeld, 2008; Gomes et al., 2017; Raghuwanshi et al., 2017]. For instance, the inhibition of ACLY (an enzyme for the production of acetyl-CoA for histone acetylation) caused a decrease in the expression of pluripotency markers (*Oct4*, *Tra1-81*) during early differentiation, and supplementation of acetate (a precursor of acetyl-CoA) completely rescues this reduction [Moussaieff et al., 2015]. This indicates that histone acetylation contributes to early differentiation in ES cells. Threonine and methionine (which are required for the synthesis of the methyl group donor [SAM: S-adenosylmethionine] for DNA and histone methylation) and their metabolism are also required to maintain pluripotency in mouse and human ES cells [Wang et al., 2009; Shyh-Chang et al., 2013; Shiraki et al., 2014; Tsogtbaatar et al., 2020] (Fig. 1).

In summary, metabolites regulate stem cells by acting as signaling factors and supplying epigenetic substances.

Starvation Causes Female-To-Male Sex Reversal in Medaka

Starvation and dietary restriction suppress the reproductive cycle in mouse, rat, cattle, sheep, and human [Van der Spuy, 1985; Schillo, 1992; Baird et al., 2006; Elias et al., 2007; Della Torre et al., 2011; Matsuzaki et al., 2011] and disrupt gonadal maintenance in nematoda, fly, zebrafish, chicken, and duck [Hosoda et al., 1955; Drummond-Barbosa and Spradling, 2001; Angelo and Van Gilst, 2009; Song et al., 2009; Fan et al., 2021]. As a result, the reproductive cycle is delayed, and non-mammal animals display a regression of the gonad. Whether starvation affects sex differentiation is not known.

Medaka employ a XX/XY sex determination system [Aida, 1921; Matsuda and Sakaizumi, 2016], however, environmental factors (green light, hypoxia, and high temperatures) cause sex reversal [Hayashi et al., 2010; Cheung et al., 2014; Hayasaka et al., 2019]. In all cases, a genetic female (XX) developed into a male without a sex determination gene (*Dmy/dmrt1bY*). These reports indicate that the sex of medaka is affected by environmental factors. Recently, we found that 5 days of starvation just after hatching caused female-to-male sex reversal [Sakae et al., 2020]. Under starvation conditions, metabolic alterations play an important role in sex differentiation. Several approaches using different types of mass spectrometry have revealed a common set of metabolic

Fig. 1. Metabolic pathways centered around acetyl-CoA. Pantothenate is metabolized to coenzyme A (CoA). Then, CoA attaches to the acetyl residue and forms acetyl-CoA which is an essential material for the TCA (tricarboxylic acid cycle) cycle, lipogenesis (fatty acids and cholesterol), and epigenetic modification. Key metabolites, enzymes, and inhibitors for starvation-induced sex reversal in medaka [Sakae et al., 2020] are shown in red letters. A star indicates rate-limiting enzymes.

pathways in XX larvae. One of them is a pantothenate pathway, which is essential for providing coenzyme A (CoA) [Lopez Martinez et al., 2014] (Fig. 1). Pantothenate is known as vitamin B5, and animals cannot produce pantothenate on their own [Lopez Martinez et al., 2014]. Starved XX larvae displayed an elevation in pantothenate and a reduction in downstream metabolites of pantothenate. This suggests that starvation suppresses the flow of pantothenate to CoA and results in a decrease in the amount of CoA. Consistent with this, a decrease in lipids (especially triacylglycerols, cholesterol, phosphatidylethanolamines, phosphatidylserines, and sphingolipids) was observed. Pharmacological inhibition by pantothenate kinase (Pank, the rate-limiting enzyme of the pantothenate pathway) inhibitor and fatty acid synthesis (the first step of lipogenesis) inhibitor (C75) were conducted to investigate whether metabolic alterations actually affect the sex (Fig. 1). Both inhibitions displayed female-to-male sex reversal (Pank: 15%, C75: 13%). This is the first report of sex reversal due to metabolic inhibition.

In order to confirm whether metabolism is involved in the mechanism of sex differentiation, we analyzed the expression of sex differentiation-related genes (*foxl2*, *aromatase*, *gsdf*, and *dmrt1*) during the period of sex differentiation. Quantitative reverse transcription PCR (RT-qPCR) analysis revealed that the expression of female differentiation-related genes (*foxl2* and *aromatase*) decreased upon starvation. In contrast, the expression level of male differentiation-related genes (*dmrt1*, but not *gsdf*) increased both under starvation and by inhibition of Pank and fatty acid synthase. Finally, starvation treat-

ment in the *dmrt1* mutant did not show any female-to-male sex reversal. Therefore, we concluded that starvation-induced sex reversal was caused by ectopic *dmrt1* expression in XX medaka larvae by metabolic alterations.

In zebrafish, a low food treatment during the larval to juvenile stage (81 days) produced a higher male ratio than high food treatment [Lawrence et al., 2008]. Starvation for 30 days in adult bivalves caused a biased sex ratio towards males compared to before starvation [Stenyakina et al., 2010]. These reports suggest that sex reversal by starvation is not limited to medaka.

Is the Alteration in the Pantothenate Pathway a Common Feature in Response to Stress?

Interestingly, the elevation of pantothenate is also observed in blood samples from starved humans (10, 34, and 58 h of starvation) [Teruya et al., 2019]. The pathway analysis based on metabolome data indicates that the pantothenate pathway is prominent under high-temperature conditions in 1-day-old chicken (*Gallus gallus*) and adult black rockfish (*Sebastes schlegelii*) [Tomonaga et al., 2018; Song et al., 2019]. These reports suggest that the pantothenate pathway is altered under stress conditions. Daphnia undergo parthenogenesis in a stable environment to form a female population. In natural conditions, a short day causes a switch from parthenogenesis to sexual reproduction. Consistent with this an increase in pantothenate was observed in Daphnia under short-day conditions. Interestingly, pantothenate treatment produced male individuals even under long-day conditions [Toyota et al., 2016].

Fig. 2. Model of female-to-male sex reversal under starvation conditions in XX medaka. Under normal feeding conditions, lipids play a role in the suppression of *dmrt1* expression in genetic female (XX) larvae. Starvation changes the metabolism (pantothenate pathway and fatty acid synthesis) and causes a decrease in lipids. Lipid deficiency stimulates ectopic expression of *dmrt1*. Finally, XX medaka develop into functional males. The circle size indicates the strength of the effectiveness of each factor. Red circles, female factors; blue circles, male factors; gray circles, unknown factors.

The study of starved medaka indicated that acetyl-CoA, a metabolite containing CoA produced by the pantothenate pathway, is an essential metabolite involved in histone acetylation, cholesterol synthesis, and fatty acid synthesis (Fig. 1). Starved XX medaka suggest that a low level of lipids due to a limited amount of acetyl-CoA causes sex reversal. In *Daphnia*, however, additional administration of pantothenate induced the production of males. In this case, acetyl-CoA might be used for the biosynthesis of juvenile hormone, a male-producing hormone, through the mevalonic acid (MVA) pathway under short-day conditions (Fig. 1). Consequently, in either case, the pantothenate pathway may play an important role in sex differentiation by modulating the supply of acetyl-CoA.

The Candidate Factors for Starvation-Induced Sex Reversal

In recent years, sex has been considered not to be determined by a single factor but by the interaction of multiple factors and is more like a “seesaw game” [Capel, 2017]. Our study of sex reversal by starvation supports the notion that metabolism and lipids are the driving factors in this seesaw (Fig. 2). In the following, we discuss the factors that have been reported to be associated with metabolism, starvation, and sex reversal: lipids, hypothalamus-pituitary-gonadal (HPG) axis, epigenetics, and germ cells.

Lipids

The definition of lipid is loose, but the International Union of Pure and Applied Chemistry (IUPAC) defines lipid as a substance of biological origin that is soluble in

nonpolar solvents [Moss et al., 1995]. They include a group of glycerolipids (triacylglycerol, phospholipid, and glycolipid), sterols (cortisol, estrogen, and androgen), and prenols (carotenoid, vitamin E, and vitamin K).

Steroid hormones have been shown to play an important role in sex differentiation [Guiguen et al., 2010; Morohashi et al., 2013], but there are no reports on other lipids affecting sex. The involvement of fatty acid synthesis in female medaka sex differentiation suggests the importance of lipids in sex differentiation [Sakae et al., 2020]. Recently, it was reported that the orphan nuclear receptor, PPAR (peroxisome proliferator-activated receptor; in which lipids are used as ligands), functions downstream of cortisol on sex reversal at high temperatures [Hara et al., 2020]. PPAR regulates gene expression in a lipid ligand-dependent manner [Grygiel-Górniak, 2014; Bervejillo and Ferreira et al., 2019]. Inhibition of fatty acid synthesis indicates decreased expression of *foxl2* and *aromatase* and increased expression of *dmrt1* [Sakae et al., 2020]. It is possible that certain lipids act as ligands and regulate the expression of these genes through orphan nuclear receptors such as PPAR to control sex differentiation.

HPG Axis

The HPG axis, which controls the reproductive cycle of vertebrates, is mainly composed of neuropeptides (e.g., kisspeptin, AgRP, and melanocortin), GnRH, LH, FSH, and sex hormones (estrogen and androgen) [Manfredi-Lozano et al., 2018]. Kisspeptin plays an important role in the regulation of the reproductive cycle through dietary conditions [Harter et al., 2018; Matsuda et al., 2019; McIlwraith and Belsham, 2020; Talbi and Navarro, 2020]. Under normal feeding conditions, kisspeptin neurons re-

ceive gonad-derived estrogen and adipocyte-derived leptin to release kisspeptin. The released kisspeptin stimulates GnRH neurons and forms a positive feedback loop [Smith et al., 2005; Qiu et al., 2011; Harter et al., 2018; Manfredi-Lozano et al., 2018]. On the other hand, under starvation conditions, kisspeptin neurons receive peripheral metabolic hormones (ghrelin and insulin) to suppress GnRH neurons, negatively controlling the reproductive cycle [Frazao et al., 2014; Harter et al., 2018]. In medaka, kisspeptin does not affect the neural activity of GnRH neurons, and chicken has lost kisspeptin and its receptors. Therefore, in these species, the reproductive cycle may be controlled through dietary conditions by a mechanism other than kisspeptin [Kanda and Oka, 2012; Nakajo et al., 2018].

The *gnrh3*-mutant zebrafish shows a male-biased sex ratio, and some XX individuals of the *fshr*-mutant medaka display a female-to-male sex reversal [Murozumi et al., 2014; Feng et al., 2020]. The analysis of the adult *fshr*-mutant female medaka indicated a reduction in *aromatase* expression and estrogen levels. Similarly, *aromatase* expression was decreased in the *gnrh3*-mutant of zebrafish larvae. Interestingly, the expression of *fshr* in XX medaka larvae is suppressed by high temperature and cortisol treatment, and the expression of *aromatase* is also decreased [Hayashi et al., 2010; Kitano et al., 2012]. These results raise the possibility that the HPG axis maintains female sex differentiation during sex differentiation.

Germ Cells

In medaka and zebrafish, germ cells play an important role in feminization [Kurokawa et al., 2007; Morinaga et al., 2007; Dranow et al., 2013; Tzung et al., 2015]. In both species, loss of germ cells in females caused the failure of maintaining the expression of aromatase in gonadal somatic cells and showed female-to-male sex reversal [Kurokawa et al., 2007; Siegfried and Nüsslein-Volhard, 2008]. In addition, half of the *dazl* mutant XX medaka, in which primordial germ cells (PGCs) fail to develop into gonocytes, exhibit female secondary characteristics [Nishimura et al., 2018]. This suggests that germ cells that are established as the cells to undergo gametogenesis are more critical for female differentiation than PGCs. Supporting this, an increase of the germ cells in male medaka at the larval stage displayed male-to-female sex reversal [Morinaga et al., 2007; Nakamura et al., 2012]. It suggests that even germ cells in genetic males have the ability of feminization and would produce an unidentified factor that promotes feminization beyond the threshold derived from the number of germ cells. Starvation-treated

Fig. 3. Model of regulation of sex differentiation by internal factors. Sex differentiation is likely affected by a comprehensive control of metabolism, germ cells, epigenetics, lipids, and/or HPG (hypothalamus-pituitary-gonadal) axis. The combined actions may regulate gene expression such as *foxl2*, *aromatase*, and *dmrt1*. Metabolism plays an important role in regulating sex by affecting other factors in response to environmental changes such as starvation, high temperature, and hypoxia. The lines connecting the factors show internal relations. The references for the relationships are described in Table 3.

XX medaka larvae displayed a decrease in germ cell numbers (unpubl. data), although this remains to be confirmed statistically.

Conclusion and Perspective

The examples described in this work still remain fragmentary and less solid in relating one mechanism to another. In recent years, several novel non-acetylsine ac-

Table 3. References for the relationship between each factor

Connection	References
Metabolisms–germ cells	Kanatsu-Shinohara et al. [2016]; Hayashi et al. [2017]; Tischler et al. [2019]
Metabolism–HPG axis	Harter et al. [2018]; Manfredi-Lozano et al. [2018]; Talbi et al. [2020]
Metabolisms–lipids	Chandel [2014]; Sakae et al. [2020]
Metabolism–epigenetics	Moussaieff et al. [2015]; Reid et al. [2017]; Tsogtbaatar et al. [2020]
Germ cells–HPG axis	Feng et al. [2020]; Meccariello et al. [2020]
Germ cells–lipids	Kurokawa et al. [2007]; Siegfried and Nüsslein-Volhard [2008]; Kitano et al. [2012]; Dranow et al. [2013]; O’Shaughnessy et al. [2014]; Tang et al. [2017]
Germ cells–epigenetics	Bao and Bedford [2016]; Tischler et al. [2019]; Kuroki et al. [2020]
HPG axis–lipids	Cui et al. [2012]; Frazao et al. [2014]; Murozumi et al. [2014]; Manfredi-Lozano et al. [2018]
HPG axis–epigenetics	Uenoyama et al. [2016]; Zhang et al. [2017]; Aylwin et al. [2019]; Shalev and Melamed [2020]
Lipids–epigenetics	Mali et al. [2010]; Tan et al. [2011]; Tsogtbaatar et al. [2020]

ylations such as butyrylation, β -hydroxybutyrylation, and propionylation have been identified as pathways that regulate gene expression [Tan et al., 2011]. This may add a novel connection between lipids and epigenetics. In addition, glycolysis, α -ketoglutaric acid, and fatty acids have been shown to be involved in germ cell fate in mouse and nematoda (connection between germ cell–metabolism, germ cell–lipids) [Kanatsu-Shinohara et al., 2016; Tang and Han, 2017; Tischler et al., 2019], and *FSHR* expression is regulated by DNA methylation in sheep (connection between HPG axis–epigenetics) [Zhang et al., 2017].

For appropriate sex differentiation, metabolism is associated with other factors such as lipids, HPG axis, epigenetics, and germ cells, and this may comprehensively regulate the expression of sex differentiation-related genes (Fig. 3; Table 3). Special attention should be paid to metabolism as it seems to be deeply involved in all factors such as lipids, HPG axis, epigenetics, and germ cells. A multifaceted approach will elucidate the mechanism of the environmental regulation of sex.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Y.S. wrote the manuscript and drew all tables and figures. M.T. supervised the content.

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