猪克隆胚胎体外培养的研究进展

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摘要 猪克隆胚胎的体外培养一直是限制克隆猪大批量生产的制约因素。早期, 猪克隆胚胎的培养主要是借鉴体外受精胚胎的体外培养体系, 但培养效果并不理想。这是因为, 两者的代谢方式不同, 其表现在两种胚胎对氧气的需要时段不同, 体外受精的胚胎在致密化阶段对氧气的消耗量增大, 而克隆胚胎是在囊胚以后的阶段, 因此, 建立更适合克隆胚胎氧需求的培养体系可有效提高克隆胚胎的发育能力。另外, 通过向基础培养液中添加各种添加剂也可改善克隆胚胎的发育。该文分别从猪克隆胚胎体外培养的研究历史与现状、影响因素(包括基础培养液、各种添加物和氧分压等)及体外培养优化策略等进行了论述, 旨在为建立稳定的猪克隆胚胎体外发育的最佳培养体系提供理论依据。

关键词 猪; 克隆; 胚胎; 体外培养

Current Progress in the Culture of Porcine Clone Embryos *In Vitro*

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Abstract The culture of porcine clone embryos *in vitro* had always restricted the massive production of clone pigs. In the earlier time, the culture of porcine clone embryos used the culture system for embryos *in vitro* fertilization; however, it was not suitable for the development of porcine clone embryos. The main reason was that two different embryos had different metabolic ways and they had different periods on oxygen consumption. It was reported that oxygen consumption became increased from the compact morula stage for the embryos *in vitro* fertilization; nevertheless, it was increased after blastocyst stage for the clone embryos. So it would greatly improve the development of porcine clone embryos to establish a suitable culture system for embryo's oxygen consumption. Furthermore, some additive agents could also improve the development of clone embryos. This paper reviewed the history and current situation on the study of culture of porcine clone embryos, the factors on culture (including of basic culture medium, various additive agents and oxygen pressure) and strategy for *in vitro* culture optimization, aiming to provide theoretical basis to establish best culture system to culture porcine clone embryos.

Keywords porcine; clone; embryo; *in vitro* culture

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我国是世界上最大的生猪生产国和猪肉消费国, 培育高产优良的猪品种是猪育种工作的当务之急。同时, 由于猪的器官在解剖、形态及生理功能上与人类的器官最相似, 所以猪被选作最佳的动物来构建异种器官移植模型和各种人类疾病模型。自从第一头体细胞克隆猪诞生以来, 科学家们就开始尝试用转基因克隆技术制作具有遗传修饰的猪。另外, 随着基因编辑技术, 如CRISPR/CAS9等的出现, 使得制作转基因克隆猪的需求加大, 但是克隆猪胚胎的体外培养一直阻碍着该技术的发展, 到目前为止, 克隆猪的胚胎在体外能发育到囊胚的比率仍然在30%以内。早期的猪克隆胚胎培养主要是借鉴体外受精胚胎的体外培养, 但由于两者的代谢方式不同, 对氧气的需要时段不同, 因此有必要去探索建立更适合克隆胚胎氧需求的培养体系, 从而提高克隆胚胎的发育能力。本文分别从猪克隆胚胎体外培养的研究历史与现状、影响因素(包括基础培养液、各种添加物和氧分压等)及体外培养优化策略三个方面进行了论述, 旨在为建立稳定的猪克隆胚胎体外发育的最佳培养体系提供理论依据。

1　猪克隆胚胎体外培养研究的历史与现状

对于猪克隆胚胎体外培养的研究要追溯到对体内受精来源猪胚胎的体外培养。早期的研究表明, 有些培养液可以支持体内来源的胚胎从四细胞发育至囊胚, 但若从一细胞胚胎开始培养, 胚胎只能在体外发育至四细胞阶段, 该现象被称之为胚胎发育阻断, 这主要与胚胎基因组激活有关[1]。为了克服这一发育阻断, 许多研究者尝试将体外受精的早期猪胚胎移植到不动情期结扎的绵羊输卵管中, 这样猪胚胎能发育至桑葚胚和囊胚阶段, 移植到代孕猪子宫中可获得仔猪[2]。之后人们发现, 在培养液中添加输卵管液或与输卵管上皮细胞共培养均能促进猪胚胎的体外发育[3]。

直到有明确配方的培养液出现, 这一僵局才被打破。比较成熟的胚胎培养液有Whitten's medium[4]、modified Kreb's Ringer bicarbonate medium[5]、NCSU23 medium[3]和Beltsville Embryo Culture Medium-3[6]等。在这些培养液中, NCSU23培养液效果最佳, 它能够支持胚胎发育到囊胚[3]。自1996年克隆羊多莉的出现以来, 很多科学家都在为克隆猪的出生而做着不懈的努力, 当时大部分实验室都用NCSU23做为核移植胚胎的培养液。2002年, Yoshioka等[7]发明了一种化学成分确定的培养液PZM3(Porcine Zygote Media 3), 并证实PZM3比NCSU23更适合猪胚胎的代谢和营养需求, 用PZM3培养的猪胚胎的囊胚率和胚胎细胞数都要高于NCSU23。为了进一步提高猪克隆胚胎的发育能力, 很多研究人员开始尝试往培养液中添加各种营养成分。比如, 在第4 d向培养液中添加10%的胎牛血清, 可以明显地提高囊胚率并且能提高解冻后冻存囊胚的成活率[8-9]。还有几种其他的添加物, 例如表皮生长因子[10]、胰岛素样生长因子[11]、粒细胞–巨噬细胞集落刺激因子[12-13]和抗氧化剂(包括维生素E[14]、维生素C[15]、花青素[16]以及3-hydroxyflavone[17])都被证明能提高克隆胚胎的体外发育能力(表1)。虽然某些添加物可以提高猪克隆胚胎的体外发育率, 但是不同添加物的作用会因胚胎的类型不同而受到影响, 并且体外发育能力的提高有时不能等同于克隆猪的出生率的提高。

另有研究表明, 克隆胚胎与体外受精胚胎具有不同的代谢方式, 主要表现在这两种胚胎对氧气的需要时段不同, 体外受精胚胎在致密化阶段对氧气的消耗增大, 而克隆胚胎对氧气的需求直到第7 d囊胚阶段才会增加[18]。多项研究发现, 克隆胚胎在低氧环境下生长较好, 这就与克隆胚胎对氧气消耗的时段需求一致[19]。已证实小鼠的克隆胚胎具有与体外受精或体内受精胚胎不同的培养需求, 克隆胚胎在体细胞培养液中比在胚胎培养液中发育要好[20]。克隆胚胎的这种特殊培养需求和代谢可能会引起异常的滋养外胚层发育[21]。当用体外受精来源的滋养外胚层替代克隆胚胎的滋养外胚层时, 小鼠克隆的成功率增高。另外, 当用体外受精来源的内细胞团结合克隆来源的滋养外胚层时会导致嵌合胚胎的发育率降低[22]。在猪的克隆囊胚中, 滋养外胚层的细胞数显著降低, 而凋亡的发生率大大增加, 这就表明未成熟的滋养外胚层细胞与猪的克隆效率低有直接关系[7,18,23-24]。

2　猪克隆胚胎体外发育的影响因素

2.1　培养液及各种添加物对猪克隆胚胎体外发育的影响

2.1.1　*基础培养液*　由于猪胚胎内含有大量的脂滴, 对温度非常敏感, 所以猪的胚胎是公认的最难

表1 各种添加物对猪克隆胚胎发育的影响

Table 1 The effect of different additive on the development of porcine clone embryos

基础培养液 Basic medium	添加成分 Additive	克隆胚胎囊胚率(%, 处理组*vs*未处理组) Blastocyst (%, treated *vs* untreated)	参考文献 Reference
NCSU23	VEGF	34.4% *vs* 23.97%	[25]
	Melatonin	20.0% *vs* 11.7%	[26]
	LPA	–	[27]
	50 nmol/L TSA	46.4% *vs* 17.7%	[28]
	37.5 nmol/L TSA	80% *vs* 54%[a]	[29]
	VPA	40.8% *vs* 23.4%	[30]
PZM3	Scriptaid	21% *vs* 9%	[31]
	Scriptaid and MG132	25.1% *vs* 18.5%[b]	[32]
	Oxamflatin	25.5% *vs* 10.3%	[33]
	vitamin C	36% *vs* 11.5%	[15]
	m-carboxycinnamic acid bishydroxamide	26.5% *vs* 12.7%	[34]
	5-aza-dC	29.64% *vs* 20.16%	[35]

a: 手工克隆; b: MG132+Scriptaid与Scriptaid比较。

a: handmade cloned; b: MG132+Scriptaid *vs* Scriptaid.

进行体外培养的胚胎之一[36]。在NCSU23发明之前, 猪胚胎在体外发育到囊胚阶段是不可能的[37]。除了NCSU23培养液外, 还有多种培养液可以提高猪胚胎体外发育的效果, 例如, modified Whitten medium[38]、modified Chatot、Bavister medium[39]和Beltsville embryo culture medium(BECM)-3[6]等都支持猪胚胎发育到囊胚。直到PZM3培养液的发明, 才有效地提高了猪胚胎的体外发育能力[7]。已证实, 体外受精的猪胚胎在PZM3中可发育至囊胚, 经胚胎移植到受体猪后可生出仔猪。与NCSU23相比, PZM3培养的猪胚胎囊胚率和胚胎细胞数都要高[19]。另外, 孤雌的猪胚胎在PZM3里培养也表现出非常好的发育效果[40]。

在目前使用比较广泛的几种猪胚胎培养液中, 如NCSU23、PZM3, 其主要成分包括水、离子、能量物质、氨基酸、蛋白质及缓冲系统等(表2)。培养液中离子的最大作用就是控制培养液的渗透压, 而渗透压对于猪的胚胎发育是至关重要的。胚胎体外发育所需的能量物质主要包括丙酮酸、乳酸和葡萄糖, 这些能量物质在不同的猪胚胎培养液中是否添加以及添加的量上存在很大的差异。在猪的雌性生殖道中, 在受精后2 d左右, 四到八细胞阶段的胚胎从输卵管移动至子宫角[41]。通过对比输卵管和子宫中的液体发现, 能量底物的浓度是不同的[42]。研究发现, 猪输卵管液中的葡萄糖水平在排卵前到排卵后这个阶段有显著的下降[43]。另外, 通过分析体外胚胎的代谢情况证明, 胚胎从一细胞发育到囊胚的这个阶段对葡萄糖的利用是增长的, 这与其他物种类似[42]。因此, 在培养的第2 d改变培养液的成分来模拟体内的环境是有利的。NCSU23培养液的发明者认为, 在葡萄糖存在的情况下, 乳酸会抑制猪胚胎的体外发育, 所以在配制NCSU23时不添加丙酮酸或乳酸, 只是添加高剂量的葡萄糖。

在过去的十几年中, 氨基酸在猪胚胎体外发育中的作用不断引起大家的注意。已证实, 在猪的输卵管液和子宫液中含有大量的自由氨基酸, 这些氨基酸能够刺激胚胎的发育[44]。但是, 在向NCSU23中添加不同浓度的氨基酸时发现, 高浓度的氨基酸对胚胎的体外发育是有害的, 但是低浓度的氨基酸却有利于胚胎的体外发育[45]。Mito等[46]也证实, 在PZM3中添加谷氨酰胺和亚牛磺酸有利于囊胚的形成, 并且发现预混的氨基酸溶液是有浓度依赖性的。其主要原因是氨基酸在胚胎体外培养过程中会降解形成氨, 氨基酸在高浓度时可能会因为氨的聚集而失去作用。另有研究报道, 在猪胚胎培养的第5 d向含有葡萄糖的PZM3中添加甘氨酸能够提高体外猪胚胎的囊胚率。

2.1.2 生长因子 近来, 一些重要的卵巢内生长因子, 如存在于卵泡液中的褪黑素(melatonin)[47]和血管内皮生长因子(vascular endothelial growth factor, VEGF)[25]都被尝试添加到猪克隆胚胎的培养液中。Pang等[26]在胚胎培养液中添加褪黑素可以提高猪

表2 两种经典的猪胚胎培养液(PZM3和NCSU23)的配方对比[7]

Table 2 The component of two classical medium for porcine embryo culture (PZM3 and NCSU23)[7]

成份 Component	PZM3	NCSU23
NaCl (mmol/L)	108.00	108.73
KCl (mmol/L)	10.00	4.78
$CaCl_2·2H_2O$ (mmol/L)	—	1.70
KH_2PO_4 (mmol/L)	0.35	1.19
$MgSO_4·7H_2O$ (mmol/L)	0.40	1.19
$NaHCO_3$ (mmol/L)	25.07	25.07
Glucose (mmol/L)	—	5.55
Na-pyruvate (mmol/L)	0.20	—
Ca-(lactate)$_2·5H_2O$ (mmol/L)	2.00	—
L-Glutamine (mmol/L)	1.00	1.00
Taurine (mmol/L)	—	7.00
Hypotauraine (mmol/L)	5.00	5.00
Basal Medium Eagle amino acid (mL/L)	20.00	—
Minimum Essential Medium nonessential amino acid (mL/L)	10.00	—
Gentamicin (mg/mL)	0.05	0.05
Fatty acid-free BSA (mg/mL)	3.00	4.00
Polyvinyl alcohol (mg/mL)	—	—
Osmolarity (mOsm)	288±2	291±2
pH	7.30±0.02	7.30±0.02

克隆胚胎的发育能力, 同时有提高囊胚总细胞数和降低囊胚细胞凋亡率的作用, 但Nakano等[48]的研究却显示, 添加褪黑素不能提高克隆囊胚率和降低胚胎凋亡率。Biswas等[25]发现, 在胚胎培养液中添加VEGF时能提高体外受精胚胎的囊胚率, 但VEGF不能促进克隆胚胎发育, 这也证实克隆胚胎和体外受精胚胎对培养体系要求存在差异。

溶血磷脂酸(lysophosphatidic acid, LPA)是迄今发现的一种最小、结构最简单的磷脂, 在多种动物细胞中具有生长因子和激素样活性[49-50], 包括细胞增殖、分化、迁移、侵袭、黏附、生存和形态发生等[51]。LPA能通过诱导ROCKs活性来提高哺乳动物细胞增殖能力[52]。另外, LPA还在雌性动物的繁殖生理中起到重要作用[53], 如LPA能促进金黄地鼠卵母细胞的胞质成熟和核成熟[54]并能影响小鼠的胚胎着床[55]。2015年, Zhang等[27]发现, LPA不但能提高猪卵细胞成熟能力而且还可以提高体外受精和孤雌激活胚胎的囊胚率和囊胚细胞数, 并且能通过提高抗凋亡基因*BCL2L1*的表达水平来降低凋亡率, 但LPA是否能提高克隆胚胎的发育能力还不得而知。

2.1.3 表观遗传修饰剂　相比之下, 一些表观遗传修饰剂和细胞促重编程因子在促进克隆胚胎的发育方面的研究则有较好的进展。细胞核重编程的程度是克隆成功的又一决定因素。克隆胚胎中普遍存在的表观遗传修饰异常可能是导致克隆效率低下和表型异常的主要原因。已发现猪的克隆胚胎比正常胚胎的DNA甲基化水平要高[7]。组蛋白去乙酰化酶抑制剂(histone deacetylase inhibitors, HDACi)能引起核心组蛋白乙酰化水平的升高从而导致染色体结构上的改变[31], 这就使得核移植后体细胞基因组的转录作用得到加强[56]。组蛋白乙酰化水平的增加诱导核小体与DNA和/或连接组蛋白(linker histones)的结合比较松散, 这种松散的染色体结构会导致发育关键基因的转录变得非常活跃[31]。表观遗传修饰剂中, 组蛋白去乙酰化抑制剂, 如曲古抑菌素(trichostatin, TSA)、Scriptaid、丙戊酸(valproic acid, VPA)、Scriptaid和5-氮杂-2′-脱氧胞苷等均可不同程度地改善克隆胚胎发育。

曲古抑菌素A(trichostatin A, TSA)源自链霉菌代谢产物, 最先作为抗真菌药物使用, 近来研究表明其具有明显的组蛋白去乙酰化抑制剂的活性。TSA诱导的组蛋白乙酰化水平的提高能够显著增强小鼠、牛、猪等物种克隆胚胎的发育能力[31]。Kishigami等[56]首次报道, 组蛋白去乙酰化酶抑制剂TSA

可提高小鼠体细胞核移植胚胎的足孕发育。同年, Rybouchkin等[57]也证实, TSA处理使小鼠克隆效率提高了5倍。Zhang等[28]以50 nmol/L TSA处理猪克隆胚胎, 结果显示, 体外培养的克隆囊胚率显著提高。Li等[29]对比了不同的处理浓度后发现, 37.5 nmol/L的TSA处理手工克隆(hand-made cloned)的猪重构胚能将囊胚率提升至80%(对照组为54%)。

丙戊酸钠(sodium valproate, VPA)是一种细胞渗透性短链脂肪酸组蛋白去乙酰化酶抑制剂, 能增强猪克隆胚胎的发育能力和维持Oct3/4的表达[58], 并可以纠正猪囊胚阶段一些异常重编程因子的表达[59]。Kim等[30]在实验中发现, 使用50 mmol/L VPA处理克隆胚胎比50 nmol/L TSA处理克隆胚胎更能提高克隆胚胎囊胚率和内细胞团细胞数。

Scriptaid作为一种高效低毒的组蛋白乙酰化酶抑制剂, 可显著提高近交系动物的NIH小型猪的克隆效率。Zhao等[31]用500 nmol/L的Scriptaid处理早期克隆胚胎14~16 h, 提高了NIH小型猪克隆胚的发育能力, 并获得14头健康后代。2009年, 朱彦宾等[60]证明, 用100 nmol/L Scriptaid处理近交系五指山小型猪克隆胚胎24 h, 显著的提高了克隆胚胎的发育能力和克隆效率(囊胚率30.4% *vs* 17.5%, $P<0.05$)。另外, 蛋白酶抑制剂MG132也被用于提高克隆胚胎的发育能力。2009年, Whitworth等[61]发现, 用MG132处理激活后的克隆胚胎2 h能提高囊胚率和妊娠率。而Mao等[62]发现, 与单独使用Scriptaid相比, Scriptaid和MG132联合使用来处理胚胎时取得的效果较差。

Oxamflatin是一种组蛋白去乙酰化酶抑制剂, 它抑制核特异性组蛋白去乙酰化的能力是Scriptaid的100倍。Hou等[33]证明, 用1 μmol/L oxamflatin处理激活后的克隆胚胎15 h能够显著提高克隆胚胎的囊胚率。Mao等[32]也得到了类似的结果, 并且证明跟Scriptaid相比, 移植oxamflatin处理后的克隆胚胎能够提高出生的仔猪数量, 减少死胎的数量。5-氮杂-2′-脱氧胞苷(5-Aza-2′-deoxycytidine, 5-aza-dC)是一种DNA甲基化抑制剂, 在DNA合成过程中掺入DNA, 能够抑制DNA甲基化转移酶1(DNA methyl-transferases 1, dnmt1)的活性从而导致DNA的低甲基化水平[63]。低甲基化的DNA可允许转录因子结合到基因启动子区域从而调控基因表达。有研究证明, 5-aza-dC能够重新激活沉默的全能型基因并且能提高核重编程效率[63]。Huan等[35]证明, 用25 nmol/L的5-aza-dC处理克隆胚胎24 h能显著提高克隆胚胎的发育能力(29.64±1.65 *vs* 20.16±2.04)。另有研究报道, 虽然用5-aza-dC处理供核细胞不能显著提高胚胎的发育能力, 但是当用5-aza-dC和TSA联合作用处理供体细胞时能显著提高囊胚发育率(25.6% *vs* 16.0%, $P<0.05$)[65]。

2.1.4 维生素　维生素C, 又称为L-抗坏血酸, 是许多动物的一种必需营养物质, 同时也是有效的抗氧化剂[66]。最近的研究显示, 维生素C可以提高小鼠和人诱导多能干细胞iPS产生效率, 促进部分重新编程iPS细胞过渡至完全重编程状态, 并且可能是通过减少细胞内抑癌基因*p53*的完全表达来提高细胞重编程效率[67]。2011年, Huang等[15]证明, 维生素C可显著改善猪克隆胚胎的囊胚率(36.0% *vs* 11.5%, $P<0.05$)和妊娠率, 并且提高组蛋白H3K5的乙酰化水平和全能基因*Oct4*、*Sox2*和*Klf4*在克隆囊胚中的表达水平。

2.2 氧分压对猪克隆胚胎体外发育的影响

胚胎培养体系中的氧分压是另一个影响胚胎发育的因素[68]。尽管很多研究组在20%的氧分压下也能成功地得到克隆囊胚甚至出生克隆动物[69-71], 但是在动物生殖系统中的氧分压是远远低于20%的。在几种哺乳动物中检测到输卵管和子宫内的氧含量大约在2%~8%之间浮动[72], 而空气中氧的含量为20%左右, 因此, 在体外培养时降低氧浓度以模拟体内培养环境有可能会提高胚胎发育的潜能。

多数的胚胎培养方法是使用20%的氧浓度进行体外培养, 但如此高浓度的氧可能会在培养过程中产生较多的活性氧(reactive oxygen species, ROS), 而ROS能够对细胞产生损伤包括DNA损伤、脂质过氧化作用、蛋白的氧化修饰等[73], 也可能会引起细胞凋亡[74]。理论上, 体外低氧可减少培养体系中自由基和ROS的形成, 而且猪卵母细胞和胚胎内含有大量的脂肪颗粒, 被ROS诱导的脂肪氧化作用会对细胞分裂、代谢产物转移和线粒体的功能产生有害影响。Booth等[75]也发现, 猪的体内受精胚胎、体外受精胚胎和孤雌激活胚胎对不同氧分压也有偏好性, 其中低氧分压能显著提高体内受精胚胎和孤雌胚胎的囊胚发育率和细胞数, 而对体外受精胚胎发育无促进作用。Nanassy等[40]认为, 猪的孤雌胚胎在低氧和高氧下的发育能力没有显著差别。Yoshioka等[7]也证明, 在NCSU23培养液中培养时, 低氧不能

提高猪体外胚胎的发育能力; 但在PZM3中培养时, 低氧能显著提高第6 d的囊胚率和第8 d的囊胚孵化率以及内细胞团数和总胚胎细胞数。Im等[19]证明, NCSU23中培养的猪克隆胚胎低氧环境下囊胚率(12.3%±1.4% *vs* 7.2%±1.4%, $P<0.05$)和细胞总数(19.4±1.0 *vs* 12.2±0.8, $P<0.05$)要显著高于高氧环境培养的, 而PZM3中培养的克隆胚胎的囊胚率和细胞总数在低氧和高氧条件下没有显著差异。以上研究说明, 不同胚胎类型在不同培养体系中结果也有所不同。

3　猪克隆胚胎体外培养的优化策略

3.1　共培养系统

共培养是指把胚胎放在输卵管上皮细胞或其他类型的辅助细胞上培养。研究表明, 共培养技术可以改善胚胎的体外培养效果, 克服体外发育阻断, 提高囊胚率和胚胎细胞数[76-78]。共培养支持胚胎体外发育可能的作用机制是: (1)共培养细胞可分泌一些对早期胚胎有利的物质, 如生长因子和糖蛋白; (2)共培养体系可以代谢降解胚胎发育过程中产生的有毒物质, 如次黄嘌呤和氧自由基; (3)共培养细胞可能通过胚胎与细胞接触促进胚胎发育。2012年, Ju等[79]证明, 将猪的克隆胚胎与卵丘细胞共培养能够显著提高克隆胚胎的囊胚率(26.6% *vs* 13.0%, $P<0.05$)并且降低胚胎的凋亡比率。但由于接触面积的有限, 胚胎的2D培养还存在局限性。

目前通过使用3D培养的方法, 细胞不仅为胚胎提供结构支持, 而且能与周围的环境形成更多的接触, 更能模拟体内的培养环境。另外, 3D培养系统中糖蛋白和一些大分子阵列的方向和植入比2D培养系统更加适合胚胎的培养需求。目前, 大部分的3D培养工作都集中在卵泡培养和卵母细胞的培养上, 为了将3D培养体系运用到胚胎培养上, 还需要对目前的体系进行优化, 增加胚胎的可视性, 从而方便对胚胎进行分级和操作。目前使用在3D培养体系中的主要成分包括胶原蛋白[80-82]、藻酸盐[83-84]、骨基质[85]和特制的琼脂糖等[86-88]。如何将3D共培养技术运用到胚胎的体外发育上, 还需要进一步的研究。

3.2　基因表达谱分析

为了更有效地提高胚胎的体外发育能力, Whitworth等[89]通过分析体外培养胚胎的基因表达谱来检测相关基因的表达。他们的结果显示, 体外培养的胚胎与体内的胚胎相比有588个基因表达失调。另有报道发现, 一种精氨酸转运蛋白——SLC7A1, 在体外培养的胚胎中表达高, 但是如果在培养液中添加精氨酸表达就降低[90]。现已证实, 在体外培养的胚胎中与细胞代谢相关的基因在表达上是上调的, 这些基因主要包括参与核苷、核苷酸及核酸的代谢的基因。该现象与胚胎静置假说是一致的, 即与相对活跃的胚胎相比, 更具有活性的胚胎代谢水平较低[91]。也有人认为, 损伤较大的胚胎中其基因的转录和翻译会要求较高的营养, 从而使它们的代谢更为活跃[92]。这与比较体内体外胚胎的氨基酸表达谱的结果是一致的, 即体外胚胎的较高的氨基酸周转与它较多的DNA损伤相关[93]。

另外, 通过对深度测序数据进行分析发现着床前猪胚胎的代谢可能是由沃伯格效应(Warburg effect, WE)驱动的[94]。沃伯格效应最早是Otto Warburg于1953年提出的, 该效应描述了肿瘤细胞特殊的产生能量的方式, 即健康细胞主要依靠线粒体氧化糖类分子释放出有用的能量, 而大多数肿瘤细胞则通过产能率相对较低的糖酵解作用为自身供能, 该作用机制不需要氧气也不需要线粒体参与。沃伯格效应导致了着床前胚胎对葡萄糖的高吸收, 并且迫使细胞使用磷酸戊糖的代谢模式, 限制使用三羧酸循环。深度测序和mRNA定量分析表明, 猪囊胚的转录谱跟沃伯格效应很相似, 即在胚胎的致密化时期抑制三羧酸循环会提高猪胚胎发育的潜力[95]。Swain等[96]发现, 猪的胚胎在着床前的发育中代谢葡萄糖。这跟其他物种形成鲜明的对比, 例如在小鼠、牛和人类的胚胎培养中, 在致密化之前是不添加葡萄糖的, 因为实验证明, 如果在致密化前添加葡萄糖会抑制胚胎的发育。Swain等[96]还发现, 体内来源的胚胎比体外培养的胚胎要代谢更多的葡萄糖。Bauer等[90]也证明, 参与葡萄糖代谢的基因在体外培养的胚胎中是下调的, 因而葡萄糖代谢是下降的, 这与Swain等的结果是一致的。PZM3培养液不含有葡萄糖, 这可能是葡萄糖代谢下降的一个解释。总之, 尽管科学家们做了很多努力来得到一个理想的培养液, 但目前来看, 体外培养胚胎的发育潜力还是低于体内来源的胚胎。因此, 理解体内与体外胚胎的生理之间的差异, 是探索更好培养条件的一个非常重要的前提。

4 结语与展望

以体细胞核移植(克隆)为基础的转基因动物技术诞生于20世纪90年代, 该技术无论是在猪的遗传改良还是各种疾病模型的构建上都展示了广阔的应用前景, 尤其近几年来基因编辑技术和体细胞核移植技术的完美结合使得对克隆猪生产的需求越来越大。但是, 自从第一头克隆猪诞生以来, 猪的克隆效率低下问题始终阻碍着克隆猪的大批量生产, 而在克隆猪生产的环节中, 克隆胚胎的体外培养至关重要。目前, 已有很多报道证实可以提高猪克隆胚胎的体外发育能力, 但大部分的报道结果仅局限于体外研究, 并且评价克隆胚胎发育好坏都是依赖胚胎发育的囊胚率、囊胚的总细胞数或囊胚中内细胞团数与滋养层细胞数的比值。由于体外培养的胚胎还不能完全反应胚胎在体内的发育状况, 比如有报道称, 一种能够提高猪克隆胚胎发育的方法在处理猪克隆胚胎后, 移植后却不能获得克隆仔猪[97], 因此在评价猪克隆胚胎发育潜力时还需要一个更好的评价手段。

虽然猪克隆胚胎的体外培养体系还没有被完全优化, 但很多实验室已经通过胚胎移植克隆囊胚而获得克隆猪。现已证实, 移植50枚或者更多的新鲜猪克隆囊胚时可以获得克隆猪[98], 甚至移植冻存的体细胞核移植囊胚都能出生动物[99]。但是, 移植体细胞核移植囊胚所出生的仔猪比率明显要低于体外受精的胚胎, 移植体外培养到囊胚的体外受精胚胎出生仔猪的比率为12.7%, 而克隆胚胎的比率为1%~5%[100]。综上所述, 通过在培养液中添加各种因子, 改变培养系统中的氧分压等手段都能显著提高克隆胚胎的发育能力, 而且体外培养到囊胚的体细胞核移植胚胎移植后也能出生动物, 这都说明体外培养技术在不断地提高, 尽管如此, 我们依然有很大的提升空间。

参考文献 (References)

1 Davis DL. Culture and storage of pig embryos. J Reprod Fertil Suppl 1985; 33: 115-24.

2 Hagen DR, Prather RS, Sims MM, First NL. Development of one-cell porcine embryos to the blastocyst stage in simple media. J Anim Sci 1991; 69(3): 1147-50.

3 Petters RM, Wells KD. Culture of pig embryos. J Reprod Fertil Suppl 1993; 48: 61-73.

4 Menino AR Jr, Wright RW Jr. Development of one-cell porcine embryos in two culture systems. J Anim Sci 1982; 54(3): 583-8.

5 Krisher RL, Petters RM, Johnson BH, Bavister BD, Archibong AE. Development of porcine embryos from the one-cell stage to blastocyst in mouse oviducts maintained in organ culture. J Exp Zool 1989; 249(2): 235-9.

6 Dobrinsky JR, Johnson LA, Rath D. Development of a culture medium (BECM-3) for porcine embryos: Effects of bovine serum albumin and fetal bovine serum on embryo development. Biol Reprod 1996; 55(5): 1069-74.

7 Yoshioka K, Suzuki C, Tanaka A, Anas IM, Iwamura S. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol Reprod 2002; 66(1): 112-9.

8 McElroy SL, Kim JH, Kim S, Jeong YW, Lee EG, Park SM, *et al*. Effects of culture conditions and nuclear transfer protocols on blastocyst formation and mRNA expression in pre-implantation porcine embryos. Theriogenology 2008; 69(4): 416-25.

9 Men H, Agca Y, Critser ES, Critser JK. Beneficial effects of serum supplementation during *in vitro* production of porcine embryos on their ability to survive cryopreservation by open pulled straw vitrification. Theriogenology 2005; 64(6): 1340-9.

10 Lee GS, Kim HS, Hyun SH, Jeon HY, Nam DH, Jeong YW, *et al*. Effect of epidermal growth factor in preimplantation development of porcine cloned embryos. Mol Reprod Dev 2005; 71(1): 45-51.

11 Kim S, Lee SH, Kim JH, Jeong YW, Hashem MA, Koo OJ, *et al*. Anti-apoptotic effect of insulin-like growth factor (IGF)-I and its receptor in porcine preimplantation embryos derived from *in vitro* fertilization and somatic cell nuclear transfer. Mol Reprod Dev 2006; 73(12): 1523-30.

12 Kwak SS, Cheong SA, Jeon Y, Hyun SH. Porcine granulocyte-macrophage colony-stimulating factor improves the *in vitro* development of cloned porcine embryos. J Vet Med Sci 2012; 74(9): 1095-102.

13 Lee K, Redel BK, Spate L, Teson J, Brown AN, Park KW, *et al*. Piglets produced from cloned blastocysts cultured *in vitro* with GM-CSF. Mol Reprod Dev 2013; 80(2): 145-54.

14 Jeong YW, Park SW, Hossein MS, Kim S, Kim JH, Lee SH, *et al*. Antiapoptotic and embryotrophic effects of alpha-tocopherol and L-ascorbic acid on porcine embryos derived from *in vitro* fertilization and somatic cell nuclear transfer. Theriogenology 2006; 66(9): 2104-12.

15 Huang Y, Tang X, Xie W, Zhou Y, Li D, Zhou Y, *et al*. Vitamin C enhances *in vitro* and *in vivo* development of porcine somatic cell nuclear transfer embryos. Biochem Biophys Res Commun 2011; 411(2): 397-401.

16 You J, Kim J, Lim J, Lee E. Anthocyanin stimulates *in vitro* development of cloned pig embryos by increasing the intracellular glutathione level and inhibiting reactive oxygen species. Theriogenology 2010; 74(5): 777-85.

17 Uhm SJ, Gupta MK, Das ZC, Kim NH, Lee HT. 3-Hydroxyflavone improves the *in vitro* development of cloned porcine embryos by inhibiting ROS production. Cell Reprogram 2011; 13(5): 441-9.

18 Sugimura S, Yokoo M, Yamanaka K, Kawahara M, Moriyasu S, Wakai T, *et al*. Anomalous oxygen consumption in porcine somatic cell nuclear transfer embryos. Cell Reprogram 2010; 12(4): 463-74.

19 Im GS, Lai L, Liu Z, Hao Y, Wax D, Bonk A, Prather RS. *In vitro* development of preimplantation porcine nuclear transfer embryos cultured in different media and gas atmospheres. Theriogenology

2004; 61(6): 1125-35.
20 Gao S, Chung YG, Williams JW, Riley J, Moley K, Latham KE. Somatic cell-like features of cloned mouse embryos prepared with cultured myoblast nuclei. Biol Reprod 2003; 69(1): 48-56.
21 Martin L, Besch-Williford C, Lai L, Cheong HT, Im GS, Park KW, *et al*. Morphologic and histologic comparisons between *in vivo* and nuclear transfer derived porcine embryos. Mol Reprod Dev 2007; 74(8): 952-60.
22 Lin J, Shi L, Zhang M, Yang H, Qin Y, Zhang J, *et al*. Defects in trophoblast cell lineage account for the impaired *in vivo* development of cloned embryos generated by somatic nuclear transfer. Cell Stem Cell 2011; 8(4): 371-5.
23 Chae JI, Lee KS, Kim DJ, Han YM, Lee DS, Lee KK, *et al*. Abnormal gene expression in extraembryonic tissue from cloned porcine embryos. Theriogenology 2009; 71(2): 323-33.
24 Koo DB, Kang YK, Park JS, Park JK, Chang WK, Lee KK, *et al*. A paucity of structural integrity in cloned porcine blastocysts produced *in vitro*. Theriogenology 2004; 62(5): 779-89.
25 Biswas D, Jung EM, Jeung EB, Hyun SH. Effects of vascular endothelial growth factor on porcine preimplantation embryos produced by *in vitro* fertilization and somatic cell nuclear transfer. Theriogenology 2011; 75(2): 256-67.
26 Pang YW, An L, Wang P, Yu Y, Yin QD, Wang XH, *et al*. Treatment of porcine donor cells and reconstructed embryos with the antioxidant melatonin enhances cloning efficiency. J Pineal Res 2013; 54(4): 389-97.
27 Zhang JY, Jiang Y, Lin T, Kang JW, Lee JE, Jin DI. Lysophosphatidic acid improves porcine oocyte maturation and embryo development *in vitro*. Mol Reprod Dev 2015; 82(1): 66-77.
28 Zhang Y, Li J, Villemoes K, Pedersen AM, Purup S, Vajta G. An epigenetic modifier results in improved *in vitro* blastocyst production after somatic cell nuclear transfer. Cloning Stem Cells 2007; 9(3): 357-63.
29 Li J, Svarcova O, Villemoes K, Kragh PM, Schmidt M, Bogh IB, *et al*. High *in vitro* development after somatic cell nuclear transfer and trichostatin A treatment of reconstructed porcine embryos. Theriogenology 2008; 70(5): 800-8.
30 Kim YJ, Ahn KS, Kim M, Shim H. Comparison of potency between histone deacetylase inhibitors trichostatin A and valproic acid on enhancing *in vitro* development of porcine somatic cell nuclear transfer embryos. *In Vitro* Cell Dev Biol Anim 2011; 47(4): 283-9.
31 Zhao J, Ross JW, Hao Y, Spate LD, Walters EM, Samuel MS, *et al*. Significant improvement in cloning efficiency of an inbred miniature pig by histone deacetylase inhibitor treatment after somatic cell nuclear transfer. Biol Reprod 2009; 81(3): 525-30.
32 Mao J, Zhao MT, Whitworth KM, Spate LD, Walters EM, O'Gorman C, *et al*. Oxamflatin treatment enhances cloned porcine embryo development and nuclear reprogramming. Cell Reprogram 2015; 17(1): 28-40.
33 Hou L, Ma F, Yang J, Riaz H, Wang Y, Wu W, *et al*. Effects of histone deacetylase inhibitor oxamflatin on *in vitro* porcine somatic cell nuclear transfer embryos. Cell Reprogram 2014; 16(4): 253-65.
34 Song Y, Hai T, Wang Y, Guo R, Li W, Wang L, Zhou Q. Epigenetic reprogramming, gene expression and *in vitro* development of porcine SCNT embryos are significantly improved by a histone deacetylase inhibitor—m-carboxycinnamic acid bishydroxamide (CBHA). Protein Cell 2014; 5(5): 382-93.
35 Huan YJ, Zhu J, Xie BT, Wang JY, Liu SC, Zhou Y, *et al*. Treating cloned embryos, but not donor cells, with 5-aza-2'-deoxycytidine enhances the developmental competence of porcine cloned embryos. J Reprod Dev 2013; 59(5): 442-9.
36 Vajta G, Zhang Y, Machaty Z. Somatic cell nuclear transfer in pigs: Recent achievements and future possibilities. Reprod Fertil Dev 2007; 19(2): 403-23.
37 Marchal R, Feugang JM, Perreau C, Venturi E, Terqui M, Mermillod P. Meiotic and developmental competence of prepubertal and adult swine oocytes. Theriogenology 2001; 56(1): 17-29.
38 Beckmann LS, Day BN. Effects of media NaCl concentration and osmolarity on the culture of early-stage porcine embryos and the viability of embryos cultured in a selected superior medium. Theriogenology 1993; 39(3): 611-22.
39 Pollard JW, Plante C, Leibo SP. Comparison of development of pig zygotes and embryos in simple and complex culture media. J Reprod Fertil 1995; 103(2): 331-7.
40 Nanassy L, Lee K, Javor A, Machaty Z. Effects of activation methods and culture conditions on development of parthenogenetic porcine embryos. Anim Reprod Sci 2008; 104(2/3/4): 264-74.
41 Hunter RH. Chronological and cytological details of fertilization and early embryonic development in the domestic pig, Sus scrofa. Anat Rec 1974; 178(2): 169-85.
42 Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo *in vivo*: Metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. Fertil Steril 1996; 65(2): 349-53.
43 Nichol R, Hunter RH, Gardner DK, Partridge R, Leese HJ, Cooke GM. Concentrations of energy substrates in oviduct fluid in unilaterally ovariectomised pigs. Res Vet Sci 1998; 65(3): 263-4.
44 Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. Theriogenology 1998; 49(1): 83-102.
45 Beebe LF, Vassiliev I, McIlfatrick S, Nottle MB. Adding essential amino acids at a low concentration improves the development of *in vitro* fertilized porcine embryos. J Reprod Dev 2009; 55(4): 373-7.
46 Mito T, Yoshioka K, Yamashita S, Suzuki C, Noguchi M, Hoshi H. Glucose and glycine synergistically enhance the *in vitro* development of porcine blastocysts in a chemically defined medium. Reprod Fertil Dev 2012; 24(3): 443-50.
47 Shi JM, Tian XZ, Zhou GB, Wang L, Gao C, Zhu SE, *et al*. Melatonin exists in porcine follicular fluid and improves *in vitro* maturation and parthenogenetic development of porcine oocytes. J Pineal Res 2009; 47(4): 318-23.
48 Nakano M, Kato Y, Tsunoda Y. Effect of melatonin treatment on the developmental potential of parthenogenetic and somatic cell nuclear-transferred porcine oocytes *in vitro*. Zygote 2012; 20(2): 199-207.
49 Jalink K, van Corven EJ, Hengeveld T, Morii N, Narumiya S, Moolenaar WH. Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. J Cell Biol 1994; 126(3): 801-10.

50 Cheng HY, Dong A, Panchatcharam M, Mueller P, Yang F, Li Z, *et al*. Lysophosphatidic acid signaling protects pulmonary vasculature from hypoxia-induced remodeling. Arterioscler Thromb Vasc Biol 2012; 32(1): 24-32.

51 van Meeteren LA, Moolenaar WH. Regulation and biological activities of the autotaxin-LPA axis. Prog Lipid Res 2007; 46(2): 145-60.

52 Hashimoto T, Yamashita M, Ohata H, Momose K. Lysophosphatidic acid enhances *in vivo* infiltration and activation of guinea pig eosinophils and neutrophils via a Rho/Rho-associated protein kinase-mediated pathway. J Pharmacol Sci 2003; 91(1): 8-14.

53 Budnik LT, Mukhopadhyay AK. Lysophosphatidic acid-induced nuclear localization of protein kinase C delta in bovine theca cells stimulated with luteinizing hormone. Biol Reprod 2002; 67(3): 935-44.

54 Hinokio K, Yamano S, Nakagawa K, Iraharaa M, Kamada M, Tokumura A, *et al*. Lysophosphatidic acid stimulates nuclear and cytoplasmic maturation of golden hamster immature oocytes *in vitro* via cumulus cells. Life Sci 2002; 70(7): 759-67.

55 Liszewska E, Reinaud P, Billon-Denis E, Dubois O, Robin P, Charpigny G. Lysophosphatidic acid signaling during embryo development in sheep: Involvement in prostaglandin synthesis. Endocrinology 2009; 150(1): 422-34.

56 Kishigami S, Mizutani E, Ohta H, Hikichi T, Thuan NV, Wakayama S, *et al*. Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. Biochem Biophys Res Commun 2006; 340(1): 183-9.

57 Rybouchkin A, Kato Y, Tsunoda Y. Role of histone acetylation in reprogramming of somatic nuclei following nuclear transfer. Biol Reprod 2006; 74(6): 1083-9.

58 Miyoshi K, Mori H, Mizobe Y, Akasaka E, Ozawa A, Yoshida M, *et al*. Valproic acid enhances *in vitro* development and Oct-3/4 expression of miniature pig somatic cell nuclear transfer embryos. Cell Reprogram 2010; 12(1): 67-74.

59 Whitworth KM, Zhao J, Spate LD, Li R, Prather RS. Scriptaid corrects gene expression of a few aberrantly reprogrammed transcripts in nuclear transfer pig blastocyst stage embryos. Cell Reprogram 2011; 13(3): 191-204.

60 朱彦宾, 龙 川, 冯 冲, 潘登科, 罗玉柱. Scriptaid处理可提高近郊系五指山克隆胚胎的发育能力和克隆效率. 基因组学与应用生物学(Zhu Yanbin, Long Chuan, Feng Chong, Pan Dengke, Luo Yuzhu. Improving the developmental potency of cloned embryos and cloning efficiency in Wuzhishan inbred pig by Scriptaid treatment. Genomics and Applied Biology) 2011; 30(3): 268-73.

61 Whitworth KM, Li R, Spate LD, Wax DM, Rieke A, Whyte JJ, *et al*. Method of oocyte activation affects cloning efficiency in pigs. Mol Reprod Dev 2009; 76(5): 490-500.

62 Mao J, Tessanne K, Whitworth KM, Spate LD, Walters EM, Samuel MS, *et al*. Effects of combined treatment of MG132 and scriptaid on early and term development of porcine somatic cell nuclear transfer embryos. Cell Reprogram 2012; 14(5): 385-9.

63 Ding X, Wang Y, Zhang D, Wang Y, Guo Z, Zhang Y. Increased pre-implantation development of cloned bovine embryos treated with 5-aza-2'-deoxycytidine and trichostatin A. Theriogenology 2008; 70(4): 622-30.

64 Yang X, Noushmehr H, Han H, Andreu-Vieyra C, Liang G, Jones PA. Gene reactivation by 5-aza-2′-deoxycytidine-induced demethylation requires SRCAP-mediated H2A.Z insertion to establish nucleosome depleted regions. PLoS Genet 2012; 8(3): e1002604.

65 Ning SF, Li QY, Liang MM, Yang XG, Xu HY, Lu YQ, *et al*. Methylation characteristics and developmental potential of Guangxi Bama minipig (Sus scrofa domestica) cloned embryos from donor cells treated with trichostatin A and 5-aza-2'-deoxycytidine. Zygote 2013; 21(2): 178-86.

66 Padayatty SJ, Katz A, Wang Y, Eck P, Kwon O, Lee JH, *et al*. Vitamin C as an antioxidant: Evaluation of its role in disease prevention. J Am Coll Nutr 2003; 22(1): 18-35.

67 Esteban MA, Wang T, Qin B, Yang J, Qin D, Cai J, *et al*. Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. Cell Stem Cell 2010; 6(1): 71-9.

68 Machaty Z, Day BN, Prather RS. Development of early porcine embryos *in vitro* and *in vivo*. Biol Reprod 1998; 59(2): 451-5.

69 Betthauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, *et al*. Production of cloned pigs from *in vitro* systems. Nat Biotechnol 2000; 18(10): 1055-9.

70 Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, *et al*. Pig cloning by microinjection of fetal fibroblast nuclei. Science 2000; 289(5482): 1188-90.

71 Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, S *et al*. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 2002; 295(5557): 1089-92.

72 Fischer B, Bavister BD. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J Reprod Fertil 1993; 99(2): 673-9.

73 Johnson MH, Nasr-Esfahani MH. Radical solutions and cultural problems: Could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos *in vitro*? Bioessays 1994; 16(1): 31-8.

74 Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW, Oh KS. Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. Hum Reprod 1998; 13(4): 998-1002.

75 Booth PJ, Holm P, Callesen H. The effect of oxygen tension on porcine embryonic development is dependent on embryo type. Theriogenology 2005; 63(7): 2040-52.

76 Gandolfi F, Moor RM. Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. J Reprod Fertil 1987; 81(1): 23-8.

77 Eyestone WH, First NL. Co-culture of early cattle embryos to the blastocyst stage with oviducal tissue or in conditioned medium. J Reprod Fertil 1989; 85(2): 715-20.

78 Pampfer S, Vanderheyden I, Michiels B, de Hertogh R. Co-culture of two-cell rat embryos on cell monolayers. *In Vitro* Cell Dev Biol 1990; 26(10): 944-8.

79 Ju S, Rui R. Effects of cumulus cells on *in vitro* maturation of oocytes and development of cloned embryos in the pig. Reprod Domest Anim 2012; 47(4): 521-9.

80 Combelles CM, Fissore RA, Albertini DF, Racowsky C. *In vitro* maturation of human oocytes and cumulus cells using a co-culture three-dimensional collagen gel system. Hum Reprod 2005; 20(5): 1349-58.

81 Vanhoutte L, Nogueira D, de Sutter P. Prematuration of human denuded oocytes in a three-dimensional co-culture system: Effects on meiosis progression and developmental competence. Hum Reprod 2009; 24(3): 658-69.

82 Vanhoutte L, Nogueira D, Dumortier F, de Sutter P. Assessment of a new *in vitro* maturation system for mouse and human cumulus-enclosed oocytes: Three-dimensional prematuration culture in the presence of a phosphodiesterase 3-inhibitor. Hum Reprod 2009; 24(8): 1946-59.
83 Kreeger PK, Deck JW, Woodruff TK, Shea LD. The *in vitro* regulation of ovarian follicle development using alginate-extracellular matrix gels. Biomaterials 2006; 27(5): 714-23.
84 Jin SY, Lei L, Shikanov A, Shea LD, Woodruff TK. A novel two-step strategy for *in vitro* culture of early-stage ovarian follicles in the mouse. Fertil Steril 2010; 93(8): 2633-9.
85 Ma S, Lin H, Miao Y, Liu X, Wang B, Dai J. The effect of three-dimensional demineralized bone matrix on *in vitro* cumulus-free oocyte maturation. Biomaterials 2007; 28(21): 3198-207.
86 Willadsen SM. A method for culture of micromanipulated sheep embryos and its use to produce monozygotic twins. Nature 1979; 277(5694): 298-300.
87 Allen WR, Pashen RL. Production of monozygotic (identical) horse twins by embryo micromanipulation. J Reprod Fertil 1984; 71(2): 607-13.
88 Vajta G, Alexopoulos NI, Callesen H. Rapid growth and elongation of bovine blastocysts *in vitro* in a three-dimensional gel system. Theriogenology 2004; 62(7): 1253-63.
89 Whitworth KM, Agca C, Kim JG, Patel RV, Springer GK, Bivens NJ, *et al*. Transcriptional profiling of pig embryogenesis by using a 15-K member unigene set specific for pig reproductive tissues and embryos. Biol Reprod 2005; 72(6): 1437-51.
90 Bauer BK, Isom SC, Spate LD, Whitworth KM, Spollen WG, Blake SM, *et al*. Transcriptional profiling by deep sequencing identifies differences in mRNA transcript abundance in *in vivo*-derived versus *in vitro*-cultured porcine blastocyst stage embryos. Biol Reprod 2010; 83(5): 791-8.
91 Leese HJ. Quiet please, do not disturb: A hypothesis of embryo metabolism and viability. Bioessays 2002; 24(9): 845-9.
92 Baumann CG, Morris DG, Sreenan JM, Leese HJ. The quiet embryo hypothesis: Molecular characteristics favoring viability. Mol Reprod Dev 2007; 74(10): 1345-53.
93 Sturmey RG, Hawkhead JA, Barker EA, Leese HJ. DNA damage and metabolic activity in the preimplantation embryo. Hum Reprod 2009; 24(1): 81-91.
94 Krisher RL, Prather RS. A role for the Warburg effect in preimplantation embryo development: Metabolic modification to support rapid cell proliferation. Mol Reprod Dev 2012; 79(5): 311-20.
95 Machaty Z, Thompson JG, Abeydeera LR, Day BN, Prather RS. Inhibitors of mitochondrial ATP production at the time of compaction improve development of *in vitro* produced porcine embryos. Mol Reprod Dev 2001; 58(1): 39-44.
96 Swain JE, Bormann CL, Clark SG, Walters EM, Wheeler MB, Krisher RL. Use of energy substrates by various stage preimplantation pig embryos produced *in vivo* and *in vitro*. Reproduction 2002; 123(2): 253-60.
97 Spate LD, Redel BK, Brown AN, Murphy CN, Prather RS. Replacement of bovine serum albumin with N-methyl-D-aspartic acid and homocysteine improves development, but not live birth. Mol Reprod Dev 2012; 79(5): 310.
98 Lagutina I, Lazzari G, Galli C. Birth of cloned pigs from zona-free nuclear transfer blastocysts developed *in vitro* before transfer. Cloning Stem Cells 2006; 8(4): 283-93.
99 Li R, Murphy CN, Spate L, Wax D, Isom C, Rieke A, *et al*. Production of piglets after cryopreservation of embryos using a centrifugation-based method for delipation without micromanipulation. Biol Reprod 2009; 80(3): 563-71.
100 Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, *et al*. Successful piglet production after transfer of blastocysts produced by a modified *in vitro* system. Biol Reprod 2002; 66(4): 1033-41.

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